

**NOVEL EXPERIMENTAL THERAPIES FOR
INTESTINAL ISCHAEMIA AND REPERFUSION INJURY**

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THESIS SUBMITTED FOR A PhD IN PAEDIATRIC SURGERY

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*I, Giorgio Stefanutti, confirm that the work presented in this thesis is my own.
Where information has been derived from other sources, I confirm that this has
been indicated in the thesis.*

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RECOGNITIONS

The following work reported in my thesis has been performed by other persons as stated below:

- Chapter 7: the retrospective review of the neonatal intensive care register to identify NEC and control patients; the retrospective collection of demographics and clinical data and the retrieval of results of blood tests relative to NEC and control patients was performed by Dr. Paula Lister (Paediatric Intensive Care Unit, Great Ormond Street Hospital for Children NHS Trust, London, UK).
- Chapter 7: preparation of haematoxylin and eosin stained sections of NEC and control patients, and evaluation of the degree of histological injury on a semi-quantitative scale was performed by Dr. Paula Lister, Dr. Mark Peters (Paediatric Intensive Care Unit, Great Ormond Street Hospital for Children NHS Trust, London, UK), and Dr. Virpi V. Smith (Department of Histopathology, Unit, Great Ormond Street Hospital for Children NHS Trust, London, UK).

ABSTRACT

Intestinal ischaemia and reperfusion (I/R) contributes to the pathogenesis of numerous clinical conditions in all age groups. Many of these diseases, including neonatal necrotizing enterocolitis (NEC), result in significant morbidity and mortality through multiple organ dysfunction, and available treatment is currently limited to supporting vital functions.

My aims were: to investigate novel therapeutic strategies such as moderate hypothermia and peroxynitrite decomposition catalyst FeTMPyP [5,10,15,20-tetrakis(*N*-methyl-4'-pyridyl)porphyrinato iron (III)] in experimental models of adult and infant intestinal I/R; and to characterise the inflammatory process in human NEC, evaluating its relationship with clinical outcome.

In an adult rat model, total-body moderate hypothermia applied throughout ischaemia and reperfusion counteracts oxidative stress in both the intestine and distant organs. This suggests that hypothermia could be beneficial as a preventative measure when intestinal ischaemia can be foreseen. However, in clinical practice therapy can usually be commenced only after ischaemia has occurred. In two sets of experiments, I showed that rescue hypothermia applied after mesenteric ischaemia improves outcome in both adult and neonatal rats, and this benefit is maintained after rewarming. Hypothermic protection could result from prevention of multiple organ dysfunction through several different pathways, including modulation of hepatic phosphoenergetics, pulmonary inflammatory infiltrate, cardiac energy metabolism, and systemic oxidative stress.

Administration of peroxynitrite decomposition catalyst FeTMPyP as a rescue therapy at reperfusion also exerts a protective effect in neonatal rats, possibly via inhibition of adhesion molecule expression, leukocyte recruitment, and lipid peroxidation in the intestine, leading to prevention of systemic oxidative stress.

In a study conducted on human specimens from neonates with NEC, tissue injury seems to be mediated via increased expression of endothelial adhesion molecules ICAM-1 and P-Selectin, leading to macrophage and neutrophil infiltration. Endothelial E-Selectin is expressed exclusively in NEC patients, and appears to be a marker of rapidly evolving disease and distant organ failure.

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LIST OF ABBREVIATIONS

AP: alkaline phosphatase
AP-1: activator protein 1
ADP: adenosine di-phosphate
AMP: adenosine mono-phosphate
ANOVA: analysis of variance
ARDS: acute respiratory distress syndrome
ATP: adenosine tri-phosphate
a-PTT: activated partial thromboplastin time
BSA: bovine serum albumin
CD41, 54, 62E, 62L, 62P, 68, 99, 106: cluster of differentiation 41, 54, 62E, 62L, 62P, 68, 99, 106
CINC: cytokine-induced neutrophil chemoattractant
CK: creatine kinase
Cl₂MBP: dichloromethylene bisphosphonate
CoA: coenzyme A
CPT: carnitine palmitoyl transferase
CS: citrate synthase
CT: computerised tomography
DAB: diaminobenzidine
DIC: disseminated intravascular coagulation
DNA: deoxyribonucleic acid
EGF: epidermal growth factor
EGR-1: early growth response factor 1
EGTA: ethylene glycol tetraacetic acid
ERK: extracellular signal-regulated kinase
ET-1, 2, 3: endothelin 1, 2, 3
ET-A, B: endothelin receptor A, B
eNOS: endothelial nitric oxide synthase
FasL: Fas ligand

List of Abbreviations

FeTMPS: 5,10,15,20-tetrakis (2,4,6-trimethyl-3,5-disulfonatophenyl)-
porphyrinato iron (III)

FeTMPyP: 5,10,15,20-tetrakis(*N*-methyl-4'-pyridyl)porphyrinato iron (III)

GALT: gut-associated lymphoid tissue

GGG: γ -glutamyl-glutamate

GSH: reduced glutathione

GSSG: oxidised glutathione

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HPLC: high-performance liquid chromatography

HRP: horseradish peroxidase

ICAM-1, 2: intercellular adhesion molecule 1, 2

IFN- γ : interferon-gamma

IgA, IgG, IgM: immunoglobulin A, G, M

IL-1 α , 1 β , 4, 6, 8, 10, 12, 14, 15: interleukin 1 α , 1 β , 4, 6, 8, 10, 12, 14, 15

IL-1ra: interleukin 1 receptor antagonist

I/R: ischaemia and reperfusion

iNOS: inducible nitric oxide synthase

JAK/STAT: Janus kinase/ signal transducer and activator of transcription

LDH: lactate dehydrogenase

LFA-1: leukocyte function-associated molecule 1

LPS: lipopolysaccharide

MAC-1: macrophage 1 antigen

MAdCAM-1: mucosal addressin cellular adhesion molecule 1

MAP: mean arterial pressure

MAPK: Map-kinase

MCP-1: monocyte chemotactic protein 1

MDA: malondialdehyde

MHC: major histocompatibility complex

MNBDH: N-methyl-4-amino-7-nitrobenzofurazan

MODS: multiple organ dysfunction syndrome

MPO: myeloperoxidase

NAD⁺: nicotinamide adenine di-nucleotide

List of Abbreviations

NADPH: nicotinamide adenine di-nucleotide phosphate
NEC: necrotizing enterocolitis
NF- κ B: nuclear factor-kappa B
NO: nitric oxide
NOS: nitric oxide synthase
nNOS: neuronal nitric oxide synthase
PAF: platelet activating factor
PBS: phosphate buffered solution
PECAM-1: platelet endothelial cell adhesion molecule 1
PSGL-1: P-Selectin glycoprotein ligand 1
PT: prothrombin time
RNA: ribonucleic acid
RNS: reactive nitrogen species
ROS: reactive oxygen species
SEM: standard error of the mean
sICAM-1: soluble intercellular adhesion molecule 1
SOD: superoxide dismutase
STATs: signal transducers and activators of transcription
TNF- α : tumour necrosis factor alpha
TT: thrombin time
VCAM-1: vascular cell adhesion molecule 1

CHAPTER 1

INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

CHAPTER 1

INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

The purpose of this introductory chapter is to provide an overview of intestinal ischaemia and reperfusion (I/R) injury, including some novel therapeutic strategies in order to provide a background to the experiments described in later chapters.

1.1 ANATOMY AND PHYSIOLOGY OF SPLANCHNIC CIRCULATION

The splanchnic circulation supplies viscera contained in the abdominal cavity, including the gastro-intestinal tract (abdominal oesophagus, stomach, duodenum, jejunum, ileum, colon, appendix, and rectum), liver, gallbladder and biliary tract, pancreas, and spleen. Arterial blood supply to these organs is delivered via the coeliac axis, the superior and the inferior mesenteric arteries. Venous blood is drained from splanchnic organs into the portal circulation and eventually into the inferior vena cava through the hepatic veins.

The splanchnic circulation accounts for 25% of cardiac output (approximately 1.5l/min), and comprises 20-25% of total blood volume (Pastores et al, 1996). In resting conditions, the liver receives approximately 100ml blood/100g tissue/min, 70% of which is coming from the portal vein, and the remainder 30% from the hepatic artery (Clemmesen et al, 1999). Blood inflow to the intestine is 50-70ml/100g tissue/min, mostly perfusing the mucosal and submucosal layers and only 30% directed to the muscularis and serosa (Lundgren, 1989). Total blood inflow to splanchnic viscera is, however, highly variable, increasing dramatically during digestion, and decreasing significantly during exercise or physically stressful situations.

Tissue oxygenation in the intestine is regulated by modulating blood flow and oxygen extraction. Adult intestinal circulation is characterised by autoregulation, with flow remaining constant despite fairly large variations in arterial inflow pressure. This phenomenon can be explained by the property of vascular smooth muscle to be modulated by transmural pressure: an increased

transmural pressure will cause vasoconstriction, and vice versa. The autoregulatory capacity of villous vasculature is even higher, and villous plasma flow can be maintained at baseline levels even when arterial blood pressure is as low as 30mmHg (Lundgren, 1974). However, intestinal vascular resistance is exceptionally low in the neonatal age, which reduces the capacity of this vasculature to respond to systemic circulatory perturbations, such as hypotension and arterial hypoxemia (Reber et al, 2002).

At rest, oxygen extraction by splanchnic organs averages only 15-20%, corresponding to 50-60ml of oxygen per minute in adults. Relative reductions in total blood inflow can be tolerated by the intestine without any functional impairment via a more efficient oxygen extraction, a situation known as non-critical low flow (Samsel et al, 1994). However, once the level of maximal oxygen extraction is reached, oxygen consumption becomes flow-dependent and any further reduction in blood flow results in a decrease in absolute oxygen consumption. At critically-low flow levels below 30ml/100g tissue/min in adults, oxygen consumption becomes inadequate to meet metabolic requirements and cells shift from aerobic to anaerobic metabolism (Desai et al, 1996).

1.2 CLINICAL CONDITIONS ASSOCIATED WITH INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

Intestinal I/R injury plays a major role in the pathogenesis of several conditions, including midgut volvulus in neonates; intussusception in infants and children; acute arterial mesenteric occlusion in adults; haemodynamic and septic shock; and surgical procedures involving arterial clamping in all age groups. I/R injury of the intestine is also one of the major factors involved in the pathogenesis of necrotizing enterocolitis (NEC) in newborns, as discussed in the following chapter.

1.3 NECROTIZING ENTEROCOLITIS

Necrotizing enterocolitis (NEC) is an acquired condition characterised by

an initial mucosal injury of the intestine that may progress to transmural bowel necrosis. NEC represents the most common life-threatening gastro-intestinal disease of neonates, accounting for 5,000-7,000 new cases each year in the United States (Reber et al, 2004). Over 90% of infants who develop the disease are born preterm, and the risk is inversely related to birth weight and gestational age (Llanos et al, 2002). The incidence of NEC in full-term infants is estimated to be about 0.05 per 1,000 live births, but is as high as 7% in newborns less than 1,500g (Lemons et al, 2001; Bolisetty et al, 2000). The number of new cases per year has remained relatively stable in the last decades despite the increase in the number of very premature infants, since improvements in neonatal care have reduced the incidence in the susceptible population.

Mortality rates from necrotizing enterocolitis are still extremely high, and range from 15% to 30%. Several studies have reported that higher fatality rates are associated with lower birth weight and gestational age (Holman et al, 1997; Luig et al, 2005). Although most cases of necrotizing enterocolitis can be managed medically, an estimated 20-40% of infants undergo surgery (Lin et al, 2006). The case fatality rate with surgical intervention is as high as 50%, and is highest for the smallest, least mature infants. Mortality for this group is related to underlying clinical status and surgical treatment. Furthermore, infants needing surgery can develop postoperative complications, including wound dehiscence, intra-abdominal abscess, and intestinal stricture, and are more prone to the development of short bowel syndrome in the long-term (Blakely et al, 2005; Henry et al, 2005).

1.3.1 Pathophysiology

Pathological examination of surgically resected specimens has shown that coagulation (ischaemic) necrosis, inflammation, and bacterial overgrowth are all almost invariably present, although with individual variability in the severity of these findings (Ballance et al, 1990). Epithelial regeneration, granulation tissue formation, and fibrosis are also visible in the majority of specimens, suggesting the presence of reparative tissue changes secondary to ongoing tissue injury of at least several days duration.

However, the true aetiology and the detailed physiological pathways leading to such tissue injury in NEC remain poorly understood despite years of research. Many theories have attempted to elucidate the full pathogenesis of this disease over the last 40 years, without success. These theories and subsequent research efforts have centred on what are felt to be the most important contributing factors: hypoxic-ischaemic injury, enteral feeds with formula milk, colonisation by pathological bacteria, and the developmental immaturity of key functions (including circulatory regulation, gastrointestinal motility, digestive ability, intestinal barrier function, and immune defence) typical of premature infants.

Immaturity of the gastrointestinal tract

The importance of gut immaturity in contributing to the pathogenesis of NEC is highlighted by the fact that this condition is almost invariably found in premature infants, and their risk for NEC remains high until the post-conceptual age of 35 to 36 weeks. The presence of an immature intestine may make it more vulnerable to potentially harmful compounds, such as hyperosmolar formulas. In addition, an impaired intestinal barrier function may predispose towards abnormal colonization by both normal enteric flora and pathogenic bacteria, instigating a local inflammatory response that would increase tissue injury.

Gastric acid and pancreaticobiliary secretions decrease the load of viable microorganisms as well as intact dietary protein antigens that reach the small intestine (Dinsmore et al, 1997). However, in premature infants both production of hydrochloric acid in the stomach (Hyman et al, 1985) and pancreatic production of proteolytic enzymes (Lebenthal et al, 1980) are impaired. Reduced activity of gastric acid and pancreaticobiliary secretions and their resulting proteolysis may increase bacterial load allowing colonization by pathogens, and suppress the hydrolysis of toxins.

Composition and production of the intestinal mucous layer is also altered in premature infants. Mucus is a complex gel that covers the surface of the villous epithelium. It is comprised primarily of water and electrolytes but also contains mucins, glycoproteins, immunoglobulins, lactoferrin and lysozyme. One of the functions of mucous gel is to contain bacteria and luminal antigens preventing

their contact with enterocytes. However, expression of mucin does not reach maturity until 27 weeks gestation (Buisine et al, 1998). Immaturity of the mucous layer may therefore enhance bacterial adherence and bacterial-endothelial interactions.

A reduced production of antimicrobial agents by specialised enterocytes may also account for an increased susceptibility to bacterial agents in premature infants. Paneth cells are secretory cells located at the base of intestinal crypts that produce various peptides, including α -defensins and lysozyme (Eckmann, 2004). These substances possess wide antimicrobial properties, and confer protection against a wide array of micro-organisms, including viruses, bacteria, fungi, and protozoa. Both the number of Paneth cells, and the production of α -defensin are reduced in human premature infants compared to adults (Mallow et al, 1996).

Gastrointestinal motility is also considerably less developed in premature infants than in term infants. A coordinated peristaltic activity is fundamental to advance enteric contents along the length of the intestinal tract, and ineffective propulsion secondary to intestinal dysmotility results in both malabsorption of dietary nutrients, and bacterial overgrowth, particularly of anaerobic species. The development of peristalsis is a late event in prenatal life, and does not reach a mature pattern until 34-35 weeks of gestation (Berseth, 1996; Berseth, 1989). It is likely that this immature motility and the resulting impaired intestinal clearance contribute to bacterial overgrowth and distension from gases that are the by-products of fermentation.

In addition, premature infants have not yet developed the ability to digest and absorb nutrients (Lebenthal et al, 1999) and incompletely digested molecules (such as acidified casein or short chain fatty acids) could contribute to intestinal injury.

Enteral feeding

NEC typically develops in infants after being fed, with less than 10% of patients who are diagnosed with NEC having never been fed before its onset (Stoll, 1994). Although the mechanism is not completely understood, enteral feeding has been reported to promote NEC by providing a favourable milieu for increased

proliferation of potentially pathogenic bacteria (Lee et al, 2003). Incomplete digestion and absorption of carbohydrates facilitates bacterial overgrowth, which in turn leads to production of carbon dioxide, short-chain organic acids, and hydrogen gas by fermentation (Kien, 1990). In the premature infant who has a relative lactase deficiency, lactose ingested in the form of milk may be converted into short chain fatty acids by bacterial fermentation in the anaerobic environment of the colon (Lin, 2004). Overproduction of short chain fatty acids can arise as a consequence of significant carbohydrate malabsorption and bacterial overgrowth in premature infants, which have been shown to lead to severe mucosal injury in experimental animals (Lin et al, 2002). In fact, a randomised study has shown that aggressive regimes of enteral nutrition in the first 10 days of life are associated with an increased incidence of NEC in preterm infants (Berseth et al, 2003). However, clinical evidence supports the advantage of minimal enteral feeding used as a trophic agent to stimulate gastrointestinal mucosal development in premature infants, possibly by potentiating gut hormones (Kliegman, 1999; La Gamma et al, 1994).

A recent meta-analysis has confirmed that feeding with breast milk reduces the risk of developing NEC in preterm and low birth weight infants, compared with artificial formulas (Quigley et al, 2007). The protective effects of human milk are mostly due to the presence of several mediators that enhance innate defences against pathogens, including white blood cells, immunoglobulins, lactoferrin, and lysozyme (Neu et al, 2005). In addition, breast milk is rich in glutamine, a fundamental metabolic substrate for intestinal mucosa that is thought to become essential during stressful times (Neu, 1996), and which is not included in standard intravenous amino acid solutions due to its poor stability in aqueous solution. Human milk also contains growth factors and hormones, including epidermal growth factor (EGF), which may directly improve gastrointestinal function by promoting gut maturity (Shin et al, 2000).

Hypoxic-ischaemic injury

The importance of ischaemic injury in the pathogenesis of NEC has long been recognised, and is confirmed by the histopathology of the disease.

Coagulation necrosis has been found to be more severe than any other findings in specimens from NEC patients, and is considered the pathological hallmark of NEC (Ballance et al, 1990). Although the exact role, mechanism, and timing of intestinal ischaemia are uncertain, recent evidence has suggested that the unique characteristics of neonatal intestinal circulation make the gut prone to ischaemic insults.

Several important haemodynamic variables undergo dramatic change during the evolution from foetal to newborn life; furthermore, the response of the newborn intestine to systemic circulatory perturbations differs from that of the adult. Many of the unique features of the newborn intestinal circulation seem designed to facilitate postnatal intestinal function.

Blood flow and oxygen delivery into newborn intestine have been shown to be more than twofold greater than during foetal life in the sheep (Edelstone et al, 1982; Holzman et al, 1985). The rise in intestinal blood flow that occurs between foetal and newborn life reflects the intense metabolic activity of the intestine in the neonatal period, as it becomes the sole site for nutrient absorption. Experiments in a neonatal pig model have shown that intestinal blood flow significantly increases between postnatal days 1 and 3 (corresponding to the neonatal period in humans), and then plateaus until postnatal day 12 (corresponding to the time of weaning from milk to solids) when it begins a progressive decline. Such changes in intestinal blood flow reflect the increased metabolic rate of the intestine in the neonatal period, and are due to a reduction in intestinal vascular resistance between postnatal days 1 and 3, followed by a progressive increase between postnatal days 12 and 30 (Reber et al, 2002). Since adrenergic nerves do not participate in setting basal vascular resistance in newborn intestine (Nowicki et al, 1991), such changes in basal intestinal vascular resistance seem to be mediated by an increased endothelial production of the potent vasodilator nitric oxide (NO) (Nankervis et al, 2001), together with a reduced myogenic response and a reduced expression of endothelin 1 (ET-1) (Reber et al, 2002), which provide constrictor tone.

Basal vascular resistance plays an important role in determining the capacity of circulation to respond to stress, and the profound differences in the intestine of newborn versus adult subjects predict that the responses of these two

age groups to systemic circulatory perturbations will also differ. In particular, the lack of extrinsic adrenergic innervation in newborn intestine might impair baroreflex and chemoreflex responses. Firstly, the intensity of the pressure-flow autoregulatory response is essentially absent in neonatal swine compared to adults, and only minimal intestinal vasodilatation is possible in response to systemic hypotension which is followed by significant decrease in oxygen delivery and tissue oxygenation (Nowicki et al, 1988a; Nowicki et al, 1992). In addition, the newborn intestinal vasculature responds in a peculiar way to different degrees of hypoxemia: mild hypoxemia (oxygen tensions of about 50mmHg) determines vasodilatation and increased gut perfusion, whereas severe hypoxemia (oxygen tensions <40mmHg) causes vasoconstriction, which is followed by tissue hypoxia (Nowicki et al, 1988b). This effect may depend on the essential role of NO in regulating vascular resistance in the newborn period, and on the limited NO production in the presence of reduced availability of molecular oxygen (Marletta, 1993; Nankervis et al, 1995).

Initial research hypothesised that exposure to one or more risk factors (such as birth asphyxia, apnoea, umbilical catheterisation, respiratory distress and ventilation, patent ductus arteriosus, indomethacin treatment, and polycythaemia) might cause intestinal ischaemia as a consequence of the “diving reflex”, a physiological adaptation present in marine mammals when blood flow is diverted from abdominal organs during hypoxia in order to preserve cerebral oxygenation (Kempley et al, 1992).

Recent studies, however, seem to suggest that the ischaemic injury observed in NEC may derive from a primary disruption of endothelial cell function resulting in an imbalance between vasodilator and vasoconstrictor mediators within the newborn intestinal circulation. In particular, an impairment of endothelial production of NO would determine vasoconstriction and subsequent reduction of intestinal blood flow instead of the expected intestinal hyperaemia typical of the early newborn period.

Endothelial cell dysfunction results in a loss of the capacity of these cells to produce NO. As previously discussed, NO is fundamental in maintaining vasodilatation in the newborn intestinal circulation, as proved by the profound

vasoconstriction that occurs in newborn, but not adult swine when constitutive NO synthesis is inhibited (Nankervis et al, 2001). A decrease in NO production has also been shown to increase the activity of vasoconstrictor mediators such as norepinephrine, angiotensin II, and ET-1 by affecting their binding affinity to cellular receptors (Cahill et al, 1995; Parekh et al, 1996), an effect present in newborn but not in adult animals (Nowicki, 1999). In addition, endothelial injury could cause loss of endothelin B receptors (ET-B) located on vascular endothelial cells, which mediate vasodilatation and are particularly abundant in neonatal life (Takasuka et al, 1994).

It has been proposed that a partial ischaemic state could initiate endothelial dysfunction in newborn intestine. A reduction of blood flow to 10% of baseline values followed by reperfusion can selectively damage intestinal endothelial cells without affecting the remaining intestinal parenchyma, and this effect is age-dependent and limited to newborn animals (Nowicki, 1996). Alternative mechanisms responsible for the initial endothelial injury include the increased production of platelet activating factor (PAF) observed in neonates with NEC (Caplan et al, 1990; Hsueh et al, 2003), bacterial translocation from the intestinal lumen into the microcirculation with subsequent cell injury by bacterial toxins, and exposure of endothelial cells to intraluminal metabolites secondary to mucosal disruption by short-chain fatty acids released by fermentation of carbohydrates in dysmotile intestine.

Recent evidence suggests that an increased arterial resistance at the level of the microcirculation could be responsible for the ischaemic changes observed in the intestine of NEC patients, possibly as a consequence of increased release of pro-inflammatory cytokines (Downard et al, 2011). Alternatively, intravascular coagulation with thrombotic occlusion of small vessels could contribute to gut hypoperfusion (St Peter et al, 2007). In both cases, ischaemia due to reduced blood flow at the level of the microcirculation is likely to be a transient and dynamic phenomenon that depends on the balance between vasoconstriction and vasodilatation, or thrombosis and thrombolysis. This would explain both the heterogeneous nature of the ischaemic insult on different parts of the gut at the same time point in individual patients, and the spontaneous improvement of the

macroscopic appearance of the bowel over time observed in many cases when a “second look” laparotomy is performed.

Bacterial colonization

Abnormal bacterial colonization plays a fundamental role in the pathogenesis of NEC. This is supported by several facts. Firstly, NEC does not occur *in utero*, but only after the gastrointestinal tract has been colonised by bacterial microflora after the first feed. Secondly, the epidemiological presence of outbreaks of NEC, as well as its decreased incidence following preventative measures, are suggestive of an infective process (Kosloske, 1994). In addition, intramural gas, one of the hallmarks of NEC, results from hydrogen production during bacterial fermentation (Hoy et al, 2000). Finally, bacteria are often isolated from the blood or peritoneal fluid of infants with NEC (Kosloske et al, 1980).

However, a direct relationship with a specific pathogen has not been established. In fact, bacteria could contribute to intestinal injury as a result of bacterial overgrowth of normal flora, or rather for the effect of pathogenic species. Early colonization of the gut by *Clostridium perfringens* is correlated with later development of NEC (de la Cochetiere et al, 2004). However, colonisation of the intestine by *Enterobacteriaceae* has also been reported to be abnormally elevated in premature infants (Hoy et al, 2000), which could contribute to the inflammatory challenge of the intestinal mucosa. Further research is needed to clarify whether bacterial species play a primary role in the pathogenesis of NEC, or contribute to amplify the magnitude of intestinal injury initially triggered by other mechanisms.

Inflammatory response

An efficient inflammatory response is necessary in order to protect the intestine from potential pathogens in the microbe-rich environment of the gut. However, an uncontrolled activation of the inflammatory response results in collateral damage caused by the release of oxidants and proteases by activated neutrophils, and other mechanisms as discussed later in this Chapter. Pathological studies in intestinal specimens from NEC patients suggest the presence of severe chronic and acute inflammation, including significant infiltration of neutrophils

(Ballance et al, 1990). Several studies suggest that the inflammatory response to physiological stimuli, including interactions with the bacterial flora, may be abnormal in the immature gut. Upon stimulation with lipopolysaccharide (LPS) and interleukin-1 β (IL-1 β), human foetal intestinal cells exhibit an 8- to 20-fold increased production of the pro-inflammatory cytokine interleukin-8 (IL-8) compared to mature human enterocytes (Nanthakumar et al, 2000). This excessive pro-inflammatory cytokine production after inflammatory stimulation has been confirmed in human organ cultures from foetuses. Similarly, IL-8 production in immature enterocytes after incubation with *Salmonella* and *Escherichia. Coli* is also greatly increased over that in adult intestinal cells (Claud et al, 2004). The potential of cord blood cells isolated from the foetal side of the placenta to produce pro-inflammatory cytokines such as IL-1 β , tumour necrosis factor-alpha (TNF- α), interleukin-6 (IL-6) after challenge with LPS has also been shown to be age-dependent, being highest in preterm infants <32 weeks gestation, and gradually lower in mild premature and term babies (Dembinski et al, 2003).

Numerous clinical studies have shown increased plasma concentrations of numerous pro- and anti-inflammatory cytokines in infants with NEC compared to age-matched controls, including IL-1 β , interleukin-1 receptor antagonist (IL-1ra), IL-8, interleukin-10 (IL-10), interleukin-18 (IL-18), TNF- α , and platelet aggregating factor (PAF), confirming the presence of an intense inflammatory response at a systemic level during NEC (Harris et al, 2005; Harris et al, 1994; Rabinowitz et al, 2001; Edelson et al, 1999). In a recent study of 127 infants, patients with confirmed NEC had higher plasma levels of proinflammatory cytokines (IL-1 β , TNF- α , IL-6) not only compared to controls, but also to age-matched infants with documented sepsis (Sharma et al, 2007). Although it is not clear whether an uncontrolled activation of inflammation could be the *primum movens* leading to intestinal injury, or in turn it is secondary to a different initiating insult, it could exacerbate the damage to the intestinal barrier, as well as promoting functional impairment of distant organs.

1.3.2 Clinical presentation

Age at presentation is inversely related to gestational age at birth, with

NEC occurring up to several weeks from birth in severely premature neonates, and full-term babies often presenting in the first few days of life (Kanto et al, 1994). Infants with NEC can present with both gastrointestinal and systemic signs. In the early stages of the disease, feeding intolerance, delayed gastric emptying, abdominal distension, vomiting or occult blood in the stools can be present. Because these early signs of the disease are non-specific, sepsis may be suspected before necrotizing enterocolitis. As the disease progresses, abdominal tenderness, ascites, abdominal wall erythema or discolouration, and gross blood in the stools may be noticed, together with non-specific signs indicative of progressive deterioration such as hyper- or hypothermia, lethargy, apnoea, respiratory distress, or poor perfusion. Intestinal perforation is usually followed by signs and symptoms of peritonitis, and profound shock. The clinical progression of the disease is extremely variable, some infants experiencing a benign disease with mainly gastrointestinal symptoms, others a catastrophic illness characterised by sudden fulminant onset with respiratory and metabolic acidosis, circulatory compromise, disseminated intravascular coagulopathy, and multiple organ failure (Hall et al, 2004). In 1978, Bell proposed a system for the uniform clinical staging of infants with necrotizing enterocolitis (Bell et al, 1978), which has been subsequently modified by Walsh and Kliegman (Walsh et al, 1986). Infants were classified as having stage I (suspect), stage II (definite), or stage III (advanced) disease, which are further subdivided depending on the presence or absence of particular prognostic factors (Table 1.1).

1.3.3 Multiple organ dysfunction syndrome in necrotizing enterocolitis

Although NEC is part of a syndrome characterised by progressive failure of multiple organ systems other than the gut, the importance of multiple organ dysfunction syndrome (MODS) in contributing to the clinical outcome of patients with NEC was first recognised only in 1994 (Morecroft et al, 1994a). In a review of 46 infants with NEC, respiratory failure was retrospectively found in over 90% of patients, renal failure in 85%, cardiovascular failure in 33% and hepatic failure in 15%. Not surprisingly, the number of organs in failure increased with the severity of the disease according to Bell's staging. Moreover, the number of

systems involved was also related to outcome, being higher in non-survivors. Similar findings were reported in a retrospective review of 50 infants with confirmed NEC (Sonntag et al, 1998). Dysfunction of at least one system in addition to the gut was present in over 80% of patients, with respiratory (76%), microcirculatory (58%), cardiovascular (54%), and hepatic (44%) functions being most frequently affected. Mortality was directly related to the number of organs in failure, and was particularly high in patients who developed capillary leak syndrome. A recent prospective study proved that both the incidence of MODS and mortality are higher in infants with NEC than in age-matched patients with proven sepsis of other origins (Sharma et al, 2007). This unfavourable outcome was accompanied by more severe endotoxaemia, suggested by higher concentrations of LPS, and by a more intense inflammatory response, as confirmed by higher levels of IL-1 β , IL-6 and TNF- α .

1.3.4 Diagnosis

Radiological imaging is essential to confirm the diagnosis in infants with clinically suspected NEC, as well as to aid in management (Epelman et al, 2007). Supine anterior-posterior abdominal X-rays are usually inspected for signs of the disease, although a horizontal view (supine cross-table lateral or a left side down decubitus) can be helpful in revealing free intraperitoneal air. Early non-specific signs include diffuse distension and asymmetric bowel gas pattern, whereas definite signs include pneumatosis intestinalis and portal venous gas. Pneumatosis intestinalis, representing intramural gas produced by pathogenic bacteria, is a pathognomonic finding and can have a linear appearance when intramural air is subserosal, or a bubbly appearance when the intramural air is submucosal. Submucosal intramural gas can be confused with stool, although radiographic evidence of colonic stool is rare in premature infants. Portal venous gas appears as a narrow, linear or branching lucency overlying the liver, representing intestinal gas absorbed into the mesenteric circulation. Typical signs indicating intestinal perforation include the “football sign” (free gas outlining the falciform ligament, showing as a radiolucent area in the shape of an American football in the epigastrium on a supine abdominal X-ray) and Rigler’s sign (clear demarcation of

both the inner and the outer bowel wall). Free intraperitoneal gas can also be seen in the sub-hepatic space or hepatorenal fossa.

Haematological studies and blood biochemistry tests can help to confirm the presence of NEC, or contribute to indicate the severity of the disease, although they are not diagnostic (Neu, 2005). Severe or persistent thrombocytopenia, neutropenia, coagulopathy, or acidosis indicate advanced disease.

1.3.5 Medical Treatment

In patients with stage I NEC, the principles of treatment include bowel rest with discontinuation of enteral feeds, bowel decompression with a large-bore nasogastric tube, and broad-spectrum antibiotics (the choice should be dictated by microbiology cultures if available). Close clinical monitoring is essential, and serial radiological imaging is recommended to exclude progression of the disease even in clinically stable patients. Adjunctive therapy includes support of cardiovascular (inotropic support, fluid resuscitation), pulmonary (oxygen, mechanical ventilation), haematological (blood product transfusion to correct anaemia, thrombocytopenia, or coagulopathy), and nutritional functions (parenteral nutrition), as well as correction of acid-base imbalance as clinically indicated. If the clinical course and results from radiological and laboratory tests remain consistent with suspected NEC, the length of medical treatment (usually 7-10 days) will be dictated by clinical judgment. Medical treatment is usually required for a longer period of time in patients with Bell's stage II, and if Bell's stage III disease is suspected or confirmed, intensive cardiovascular and respiratory support is usually required and surgical intervention should be considered (Hall et al, 2004).

1.3.6 Surgical treatment

Despite recent advances in medical treatment, over 40% of neonates with confirmed NEC require surgical intervention in the acute stage (Butter et al, 2002). The presence of pneumoperitoneum, indicating intestinal perforation, is universally considered an absolute indication for surgical intervention (Pierro, 2005). Progressive deterioration of the clinical status despite aggressive medical

support over 12-24 hours, or the finding of a palpable mass with erythema of the abdominal wall in a patient with persistent intestinal obstruction or sepsis usually also warrants a recourse to surgery. A positive paracentesis is also a valid objective indication for operation in infants with NEC, although it is rarely performed (Ricketts, 1986). Other findings, including a fixed, dilated loop of bowel on serial abdominal radiographs, evidence of portal venous gas, or a positive peritoneal culture are regarded as relative indications for surgery.

The decision to operate is often difficult, and usually requires close consultation between neonatologists and surgeons. Particularly problematic is the case of infants with suspected NEC who become critically ill despite the lack a specific indication for surgery, such as pneumoperitoneum. In these circumstances, the potential benefits of an operation need to be weighed against the morbidity and possible mortality associated with surgical procedures. Several different options have been described for acute NEC, and the choice of surgical procedure is contentious. The usual surgical approach consists in a laparotomy. The aims of such approach consist in removing all segments of clearly necrotic intestinal segments in order to control sepsis, and at the same time preserving as much bowel length as possible to prevent short bowel syndrome. These principles explain why the decision-making process during laparotomy largely depends on the extent of the disease encountered, as well as on the clinical stability of the patient.

If the disease is focal with necrosis limited to a single intestinal segment, resection of the affected segment and creation of one or more enterostomies can be performed (Muscarella et al, 1987). This option can be advantageous in unstable patients that cannot withstand a longer and more complex procedure. In addition, diverting the bowel allows adequate rest for the distal gut in the acute phase of the disease. However, the presence of an enterostomy can be associated with fluid and electrolyte imbalances, particularly for proximal stomas, as well as with difficulties in intestinal absorption once enteral feedings are restarted. In addition, complications inherent to the presence of one or more stomas (including prolapse, stenosis, and maceration of the surrounding skin) are frequently encountered, that may require re-operation (O'Connor et al, 1998). Even in the absence of any complication, a second operation is anyway required for the closure of the stoma.

Primary resection of the necrotic gut and anastomosis has been proposed in order to overcome these potential drawbacks (Ade-Ajayi et al, 1996), although fashioning the intestinal anastomosis in a potentially inflamed and ischaemic bowel is of concern. Initially restricted to patients in good general conditions and with localised disease, this procedure has been extended to micro-premies, patients with severe NEC or with multifocal disease (Hall et al, 2005; Fasoli et al, 1999), for whom multiple resections and anastomoses may be required. Although no conclusive evidence has been provided on the superiority of either approach, primary intestinal anastomosis has been shown to be a valid alternative to enterostomy (Fasoli et al, 1999).

If a multifocal form of disease is encountered at laparotomy, with several different segments of the intestine appearing necrotic, a “clip and drop” technique can be applied (Vaughan et al, 1996). After resection of frankly necrotic bowel segments, the ends are clipped and the abdomen is closed. This minimises the duration of the surgical procedure in an unstable patient, and is followed by a “second look” laparotomy after 48-72 hours, at which time a definitive procedure is performed salvaging as much bowel as possible. If pan-intestinal NEC is found, with more than 75% of the bowel appearing gangrenous, a proximal jejunostomy can be performed. The definitive procedure is then performed after 6-8 weeks in an attempt to allow both improvement of general conditions, and partial healing of the affected gut. Although a good survival rate has been reported with this technique (Sugarman et al, 2001), not removing the necrotic intestine increases the risk of development of a progressive systemic inflammatory response and sepsis due to bacterial translocation.

In 1977, Ein et al. proposed the insertion of a peritoneal drainage as a definitive treatment for premature newborns with perforated NEC (Ein et al, 1977). This was initially designed as a temporary solution to allow drainage of air and faecal material and therefore relieve intra-abdominal pressure in patients too unstable to undergo laparotomy. Although some infants improve to such an extent after insertion of a drain that a laparotomy is no longer necessary, others require a laparotomy over the following 12-24 hours. A recent multicentre controlled trial where 117 preterm infants with birth weight <1,500g and evidence of perforated

NEC were randomised to primary peritoneal drainage or laparotomy failed to show any clear benefit of either approach (Moss et al, 2006). In a similar randomised controlled trial conducted at Great Ormond Street Hospital for Children NHS Trust on 69 infants weighing <1,000g, Rees et al. confirmed that clinical outcome was similar in patients initially randomised to receive either laparotomy or peritoneal drain (Rees et al, 2008). However, 74% of patients initially treated with a drain required delayed laparotomy, suggesting that peritoneal drain is ineffective as either temporising measure or definitive treatment, and that a timely “rescue” laparotomy should be considered after initial insertion of a drain.

1.3.7 Prevention

Widely accepted strategies for the prevention of NEC include promotion of human milk feeding, and adoption of conservative feeding practices (Reber et al, 2004). A number of other strategies have been recently investigated, including probiotics, oral antibiotics, immunoglobulin supplementation, and prenatal steroids.

The protective effects of human milk versus formula feedings are well known. Two large prospective studies have found a lower incidence of NEC in preterm infants fed with their own mother’s expressed breast milk compared to those fed with formula milk (Lucas et al, 1990; Schanler et al, 1999). A recent meta-analysis showed that donor human milk reduces the incidence of NEC compared with formula milk in preterm infants (Boyd et al, 2007).

Since bacterial colonisation plays a crucial role in the pathogenesis of NEC, the use of probiotics to counteract pathogenic bacteria appears to be a promising therapy. Probiotics are defined as living microorganisms, which upon digestion in sufficient numbers exert health benefits beyond basic nutrition (Bourlioux et al, 2003). A recent meta-analysis of 12 randomised controlled trials showed that supplementation with various probiotics (including *Lactobacilli*, *Bifidobacterium*, and *Saccharomyces*) can reduce the incidence of NEC as well as improve outcome in premature infants (Deshpande et al, 2007). However, the type and dose of bacteria used, the age at initiation of the treatment as well as its

duration were profoundly different in the individual studies, and further studies are needed to delineate an ideal protocol offering the best protection.

Several studies have investigated the administration of oral IgG (immunoglobulin G) or an IgG and IgA (immunoglobulin A) combination in preterm infants in order to reduce the incidence of NEC. However, a meta-analysis of three randomised trials including a total of 2,095 neonates failed to show a significant reduction in the incidence of NEC, surgery, or mortality (Foster et al, 2004).

Prophylactic enteral antibiotics have been shown to reduce both the incidence of NEC and NEC-related mortality in a meta-analysis of five randomised trials (Bury et al, 2001). However, enteral antibiotic prophylaxis increased the incidence of colonisation with resistant bacteria, and larger studies are needed to address this issue before routine antibiotic prophylaxis is recommended.

1.3.8 Outcome

Despite the advances in medical and surgical management of infants with NEC, mortality rates are still high and ranging from 15 to 30% (Holman et al, 2006; Luig et al, 2005).

1.3.9 Animal models of necrotizing enterocolitis

Several different animal models of NEC have been developed to date, although none of them represents an ideal model in terms of consistent reproducibility, limited labour- and time-intensity required, and presence of a significant developmental component (i.e. manifesting characteristic hallmarks of NEC at the appropriate age).

Animal models based on occlusion and deocclusion of the superior mesenteric artery produce biological and pathogenetic changes characteristic of NEC. The most extensively studied is a model that utilises adult rats, where mesenteric occlusion is usually performed for 30-90min. However, the effects of blood flow impairment are more severe in developing rats as compared to adults. In weanling (90g) rats, occlusion of the superior mesenteric artery for only 1 minute carries a mortality of over 60% at 1 week, with almost half the animals

presenting signs of intestinal necrosis (Dalsing et al, 1983). In 10-day old rats, intestinal permeability is increased after only 10min occlusion of the superior mesenteric artery, followed by 30min reperfusion (Langer et al, 1993). A model of mesenteric occlusion-deocclusion has also been described in piglets. Interestingly, the severity of intestinal injury after 48 hours of mesenteric occlusion is inversely related to maturity of the experimental animals, low birth-weight (700-1,200g) animals presenting the maximal degree of mucosal injury, normal birth-weight (>1,500g) piglets showing less severe damage, and 3-month old animals proving much more resilient to mesenteric ischaemia (Sibbons et al, 1988).

The main advantage of this method is the high reproducibility of the necrotic lesions to the ileum, as well as the facility to modulate the severity of the intestinal injury by adjusting the duration of ischaemia and reperfusion. In addition, the relationship between immaturity and susceptibility to the ischaemic insult mimics the pathophysiology of human disease. However, the pathogenesis of NEC arises from multiple mechanisms, and a model based on an impairment of blood flow may appear somehow simplistic.

Systemic administration of different agents, such as TNF- α , PAF, and LPS alone or in various combinations, have been used to mimic clinical NEC. PAF is an endogenous phospholipid mediator produced by enteric bacteria (including *Escherichia Coli*) as well as inflammatory cells, endothelial cells, and platelets (Caplan et al, 2005). Intravascular administration of PAF induces rapid development of intestinal necrosis at doses as low as 2.5 μ g/kg (Gonzalez-Crussi et al, 1983), and profound and irreversible shock at higher doses. In this model, necrosis develops in the jejunum and in the caecum, but especially the distal ileum, beginning at the villous tip but sometimes extending to the submucosa or even the serosa. Systemic administration of LPS and TNF- α (Sun et al, 1988) alone can also induce similar alterations, but only for doses as high as 1mg/kg and 5mg/kg respectively. However, low (0.5mg/kg) doses of LPS act synergistically with low doses of PAF to produce significant bowel injury (Hsueh et al, 1986a). Similarly, contemporary administration of low doses of TNF- α (0.5mg/kg) and LPS (200mg/kg), causes profound shock and severe intestinal necrosis in rats (Hsueh et al, 1986b).

Although this model is highly reproducible, dose-dependent and therefore adjustable, and easy to setup, it presents some clear limitations. Firstly, it lacks any elective specificity for developing animals. In addition, intestinal injury does not develop in the totality of experimental animals. Finally, it is based on the activation of single pathogenic pathways, which does not entirely reflect the complexity of human disease.

A more sophisticated model based on various combinations of administration of enteral feeds, whole-body hypoxia, and cold stress has also been described. The original description of this model comprised a complex stress where newborn rat pups were removed from their mothers, stressed briefly with asphyxia, colonised with gram-negative enteric bacteria, and fed with artificial formula (Barlow et al, 1974). After three days, animals presented abdominal distension and discolouration, bloody stools, and respiratory distress, and intestinal pathology revealed the presence of severe necrosis very similar to that of human NEC. Asphyxia alone (incubation in 100% CO₂ for 5min followed by 100% O₂ for 5min) has also been reported to induce intestinal injury in newborn rats, although the severity of the lesions are highly variable, ranging from superficial epithelial damage with villous shortening to transmural necrosis (Okur et al, 1995). Formula feedings alone also induced moderate or severe intestinal injury resembling NEC in 70% of neonatal rats (Nadler et al, 2000). Combinations of hypoxia and formula feedings by gavage (Ergun et al, 2007); hypoxia and cold stress (Dvorak et al, 2002); hypoxia, formula feedings, and cold stress (Caplan et al, 1999) have also shown to induce intestinal injury in newborn rats.

The main theoretical advantage of these models, especially the ones combining formula feedings and hypoxia, is that the mechanism of injury mimics some of the pathogenic factors known to play a role in human NEC. However, models involving gavage feeding of neonatal rats are technically complex and therefore prone to variability. In addition, a major limitation in models involving the use of formula feedings by gavage is the induction of severe weight loss (as high as 50% of birth weight after 3 days) (Ergun et al, 2007) in the experimental animals, which adds the component of severe malnutrition to the pathogenic mechanism. Finally, these models are scarcely reproducible, as proved by the fact

that only part of experimental rats develop intestinal injury, and that the degree of intestinal injury itself is extremely variable.

1.4 MIDGUT VOLVULUS

The term midgut volvulus refers to the twisting of the portion of intestine supplied by the superior mesenteric artery (from the duodeno-jejunal flexure to the mid-transverse colon) around its vascular axis (Millar et al, 2003). Although midgut volvulus has been described in the absence of malrotation, the anatomical prerequisite for its development is the presence of an abnormal rotation and fixation of the intestine. Malrotation arises from the failure of the normal process of fixation and rotation of the intestine and the mesentery to the retroperitoneum, which takes place between the 10th and 12th gestational week. As a consequence, the base of the mesentery is particularly narrow and the ascending and descending colon are not attached to the retroperitoneum, which can cause the midgut to twist clockwise around the superior mesenteric vessels. Twisting of the mesentery results not only in an obstruction to arterial flow, but also in an impairment of venous and lymphatic drainage in the superior mesenteric territory.

Midgut volvulus is typical of the neonatal age: over 40% of cases are diagnosed in the first week, 60% in the first month, and 90% within the first year of life (Aiken et al, 2005). Clinical features include bilious vomiting, abdominal distension, pain, and bloody stools, typically followed by sepsis and progressive deterioration of vital functions. A contrast study of the upper gastrointestinal tract is the most effective radiological evaluation, which typically shows an obstruction at the level of the third portion of the duodenum (possibly with a distal spiral break or corkscrew appearance), and the duodeno-jejunal junction and the proximal jejunal loops shifted to the right of the midline (Strouse, 2004).

Once the diagnosis has been established, urgent surgical correction is mandatory because progression to intestinal necrosis carries a high risk of possible short bowel syndrome or death. The volvulus is detorted (usually in a counter-clockwise fashion), therefore re-establishing blood flow to the midgut (reperfusion). Segments of intestine appearing frankly necrotic after derotation

may need to be resected. The pedicle of the mesentery is then dissected and its base is broadened in order to reduce the probability of recurrence.

Results after surgical correction are usually excellent in the absence of necrotic bowel at the time of the procedure. However, in the presence of intestinal necrosis, mortality can be in excess of 40% in the acute phase, and short bowel syndrome can develop in the long-term (Messineo et al, 1992).

1.5 INTUSSUSCEPTION

Intussusception is the invagination, or telescoping, of a proximal portion of intestine (*intussusceptum*) into the adjacent distal segment (*intussusciens*). Increasing compression of the *intussusceptum* and its blood supply as it progresses through the distal bowel causes tissue oedema due to lymphatic and venous obstruction, resulting in arterial insufficiency with ischaemic bowel necrosis.

The vast majority of intussusceptions are ileocolic, the terminal ileum passing through the ileocaecal valve into the ascending colon. Intussusception is usually idiopathic, but in up to 12% of cases may arise from an anatomic lead point (Meckel's diverticulum, the appendix, carcinoid or lymphoid tumours and others) (Meier et al, 1996). Although it can occur at any age, the greatest incidence occurs in healthy infants between 3 and 9 months of age (Stringer et al, 1992). Infants typically present with sudden colicky abdominal pain in a previously comfortable baby. Vomiting and blood-tinged stools (sometimes with a "red currant jelly" appearance) are also often reported. Later in the course, patients appear lethargic between episodes of pain, and in the absence of appropriate treatment the outcome is often fatal as a result of hypovolaemia and sepsis (Meier et al, 1996).

An urgent ultrasound scan or a contrast enema are mandatory in every case where the diagnosis is suspected, and treatment can be attempted by hydrostatic or air reduction during the same session. Success rate of these non-invasive techniques has increased to over 75% in most centres (Stringer et al, 1992), but surgery is still advocated in case of failure of non-operative reduction or when signs of shock or peritonitis are present. Prompt recognition and adequate

treatment have dramatically improved outcome in modern practice, and survival approaches 100% in recent series (West et al, 1987).

1.6 ACUTE MESENTERIC ARTERIAL OCCLUSION

Acute mesenteric ischaemia is an often lethal condition characterised by an acute occlusion of the superior mesenteric artery or one of its major branches, and typically affects individuals of advanced age. The majority of cases of arterial occlusion result from emboli or arterial thrombi (Stoney et al, 1993). Embolisation to the superior mesenteric artery is the most frequent cause of mesenteric ischaemia, accounting for the majority of cases. Embolism is particularly frequent in patients with recent myocardial infarction, congestive heart failure, or arrhythmias. Emboli usually become lodged at major branch points within the superior mesenteric artery, typically just beyond the origin of the middle colic artery. In these cases, proximal mesenteric perfusion may be maintained resulting in a clear demarcation of the affected intestinal segment. Thrombosis of the superior mesenteric artery accounts for 25% of cases of acute mesenteric ischaemia. Arterial thrombi arise from atherosclerotic occlusive lesions, tend to occur at the origins, or very proximal segments, of the mesenteric arteries, and generally result in ischaemia of more extensive segments of bowel. In a minority of patients, ischaemia is due to nonocclusive disease or mesenteric venous thrombosis (Trompeter et al, 2002).

In recent clinical series of acute mesenteric ischaemia, mortality rates remain as high as they were 40 years ago, and vary between 60% to 100%, depending on the source of obstruction (Chang et al, 2006). Diagnosis before the occurrence of intestinal infarction is the most important factor in improving survival for patients with this condition, which highlights the importance of a high index of suspicion.

The classic clinical presentation of patients with embolic disease is of sudden excruciating abdominal pain, which is out of proportion to physical examination findings. Only a minority of patients present with the classic triad of abdominal pain, fever and blood in the stools. These patients often have a history

of cardiac disease which might predispose to embolus formation, such as atrial fibrillation or mitral valve disease. Patients with thrombotic disease often report a history of chronic abdominal pain and coexistent atherosclerotic disease. Venous thrombosis of the visceral vessels can also precipitate an acute ischemic event (Chang et al, 2006). Impaired venous return leads to interstitial swelling of the intestinal wall, with subsequent compromise of arterial flow and eventual necrosis. Etiologic factors for venous thrombosis include portal hypertension, intrabdominal sepsis, cirrhosis, pancreatitis, malignancy and trauma.

Nonocclusive mesenteric ischemia usually presents in a clinical setting of extremely low-flow splanchnic circulation, often in the presence of vasopressor use (Howard et al, 1996). No vascular occlusion is usually demonstrated because pulsatile blood flow is present in larger arteries. Patients with severe cardiac failure are at risk for nonocclusive mesenteric ischemia from vasospasm related to elevated sympathetic activity or hypovolemia.

Multiple organ failure is commonly seen in this patient population, which is the major determinant of mortality (Harward et al, 1993).

The presence of leukocytosis, acidosis, and elevated amylase, creatine kinase (CK) or lactate dehydrogenase (LDH) levels are neither specific nor sensitive. High resolution abdominal computerised tomography (CT) scan with contrast is very accurate and specific in delineating not only the extent of visceral ischemia, but also the status of flow in the mesenteric vasculature (Cikrit et al, 1996). Both mesenteric arterial and portal venous blood flow are accurately assessed with contrast CT scanning (Ridley et al, 2001). Findings of abnormal intestinal wall blood flow, arterial intraluminal defects, or a halo sign in the mesenteric veins are very well demonstrated and accurately diagnose the presence and the causes of mesenteric ischemia. Visceral CT angiography has largely replaced catheter angiography in the work up of acute mesenteric ischemia. Although catheter angiography is somewhat more accurate, it is time consuming and does not produce significantly more useful results than those of high resolution scans with coronal, sagittal, and three-dimensional reconstructions.

The traditional approach to successful management of acute mesenteric ischemia from major arterial occlusion is prompt surgical therapy. Embolism in the

superior mesenteric artery is retrieved through a transverse incision in the main trunk of the artery in the mesentery (Bjorek et al, 2002). Balloon embolectomy catheters should also be used proximally and distally, and distal flushing with heparinised saline should be performed. The treatment of choice for proximal mesenteric arterial thrombosis is aorto-mesenteric bypass (Sise, 2010). Numerous types of bypasses have been described, including antegrade supra-celiac aorto-mesenteric bypass and retrograde iliac artery to mesenteric artery bypass. Once flow has been re-established, the bowel should be reassessed for signs of restored perfusion; injection of papaverine into the superior mesenteric artery is a helpful adjunct to reduce vasospasm. Obviously necrotic bowel should be resected by stapling and dividing at healthy margins. Anastomosis of bowel segments should be deferred until reoperation at 24 to 36 hours. Temporary abdominal wall closure should be performed, followed by early anticoagulation with intravenous heparin in order to prevent re-thrombosis of mesenteric vessels.

Mesenteric venous thrombosis leading to ischemic bowel is much more difficult to manage, and the only choice is systemic anticoagulation coupled with resection of necrotic bowel (Sise, 2010). This condition often requires extensive bowel resection and survivors are left with shot-gut syndrome.

Alternative approaches to open surgery include endovascular therapy and intraarterial thrombolysis (Chang et al, 2006). Intraarterial injection of fibrinolytics in the superior mesenteric artery has been shown to have promising initial results, and in many centres it has become the initial treatment of choice for mesenteric thromboembolism.

In cases of nonocclusive ischemia, intraarterial vasodilator therapy with papaverine has been largely responsible for the decrease in mortality from 70% in the 1980s to 50%-55% during the last decade (Chang et al, 2006).

1.7 CIRCULATORY SHOCK

Circulatory shock is defined as a state of organ hypoperfusion inadequate to meet metabolic demands, with resultant cellular dysfunction and death. Mechanisms may involve decreased circulating volume (hypovolaemic shock due

to haemorrhage, burns, prolonged vomiting or diarrhoea, diabetes and others); decreased cardiac output (cardiogenic shock resulting for instance from myocardial ischaemia, arrhythmias, or pulmonary embolism); uncontrolled vasodilatation with inadequacy of intravascular volume (distributive shock such as in anaphylaxis or severe brain or spinal injuries); and reduced venous return (obstructive shock such as in pericardial tamponade or pulmonary thromboembolism).

The intense sympathetic adrenergic stimulation that characterises circulatory shock ensures that vital organs are perfused by diverting blood supply from the splanchnic circulation, including the liver (that can redistribute up to 1 litre of blood under cardiovascular stress) and the gut (Chien, 1967; Price et al, 1966; Vatner, 1974). Interestingly, splanchnic blood flow has been shown to be consistently impaired not only during hypovolemic shock, but also up to 1 hour after restoration of normal systemic haemodynamics (Edouard et al, 1994). These findings suggest that sustained splanchnic vasoconstriction develops as a consequence of a transient normotensive hypovolaemia, despite adequate treatment with fluid resuscitation and restoration of normal arterial pressure and cardiac output. This phenomenon of disproportionate splanchnic vasoconstriction appears to be mediated by both autonomic (neurally mediated adrenergic vasoconstriction) and local factors (eicosanoids, nitric oxide inhibition). The most important mechanism, however, appears to reside in a markedly greater effect of circulating vasopressor agents in the mesenteric bed compared to the systemic circulation, in particular angiotensin II (Toung et al, 2000; Suvannapura et al, 1988; Aneman et al, 2000) and vasopressin (McNeill et al, 1970; Hock et al, 1984). This state of hypoperfusion that outlasts the period of insult is the base of cellular changes causing mucosal gut damage, resulting in the loss of the intestine's barrier function allowing bacterial translocation, and in the generation of a systemic inflammatory response eventually leading to multiple organ dysfunction (Biffl et al, 1996a).

Common symptoms in patients with cardiocirculatory shock include altered mental status, tachycardia, hypotension, and oliguria; other symptoms depend on the underlying disease or secondary organ failure. Diagnosis is mostly based on clinical evidence of insufficient tissue perfusion with signs of compensatory mechanisms (such as tachycardia, tachypnoea, and diaphoresis). Primary treatment

focuses on restoring adequate perfusion to provide sufficient oxygen delivery to tissues, and supporting vital functions in case of secondary organ compromise. Untreated cardiocirculatory shock is usually fatal; even when appropriately treated, acute mortality from cardiogenic shock following myocardial infarction, for example, is as high as 25% (Jollis et al, 1996).

1.8 SEPSIS

Sepsis is characterised by a whole-body inflammatory state caused by systemic infection, mostly due to bacterial or fungal micro-organisms. Due to its high incidence (240.4 per 100,000 population per year), significant morbidity, and mortality rates of approximately 18% in the United States, sepsis represents a critical medical challenge as well as a substantial health care burden (Martin et al, 2003).

Unlike in circulatory shock, total splanchnic blood flow does not appear to be decreased, but rather increased during sepsis (Dahn et al, 1987; Wilmore et al, 1980). This may reflect the increased metabolic demand in the early, hypermetabolic stages of sepsis when overall oxygen consumption in the splanchnic organs is increased (Ruokonen et al, 1993). However, abnormal capillary blood flow distribution with decreased perfusion in the microcirculation of all layers of the intestinal wall, including villi, has been described following inoculation of *Escherichia Coli* or endotoxin (Theuer et al, 1993; Drazenovic et al, 1992). These changes are associated with impaired tissue oxygenation and cellular ischaemia, as indicated by a rise in the mucosal-arterial PCO₂ gap (Mayer et al, 1999), and by the increased catabolism of high-energy purine compounds (Schmidt et al, 1997) despite the maintenance of mesenteric oxygen delivery during experimental sepsis. Such microvascular failure is thought to be due to endothelial dysfunction following initial hypoperfusion, trauma or even direct endotoxaemic damage (Wort et al, 1999; VanderMeer et al, 1995).

The clinical presentation is often characterised by the presence of symptoms related to the underlying infectious process in addition to those typical of systemic inflammation (such as fever, tachycardia, tachypnoea, and

leukocytosis). A prompt diagnosis followed by initiation of the appropriate therapy before the onset of the hypodynamic phase is an essential factor to prevent unfavourable outcome. Treatment consists of aggressive fluid replacement, appropriate support for organ dysfunction, and eradication of the infection.

1.9 PATHOPHYSIOLOGY OF INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

Ischaemia and reperfusion injury is a complex phenomenon, and its pathogenesis, although not completely understood, is certainly multifactorial. Evidence suggests that tissue and cell alterations initiated during the ischaemic time are aggravated upon reperfusion through a series of interconnected pathways (Molls et al, 2004).

1.9.1 Ischaemia

Intestinal cells are easily injured by episodes of ischaemia, and the intestine is probably the most sensitive to ischaemic injury among the internal organs (Granger et al, 1986; Yamamoto et al, 2001). When the blood supply to a tissue is discontinued, a sequence of chemical events is initiated that leads to cellular dysfunction, oedema, and ultimately death. Oxygen as a basic fuel is crucial to cell function, and lack of its supply to tissue results in a shift from aerobic to anaerobic metabolism (Grace, 1994). Energy efficiency of anaerobic glycolysis is considerably lower than that of oxidative phosphorylation, with only 2 molecules of adenosine tri-phosphate (ATP) yielded per molecule of glucose, compared to 38 for aerobic metabolism. As a consequence, energy production for vital cell functions can be sustained for only a short period of time, which varies from minutes to hours depending on cell type (Chervu et al, 1989). Prolonged ischaemia is followed by depletion of cellular energy stores, particularly ATP, as well as by progressive acidosis due to increasing levels of by-products of anaerobic glycolysis such as lactate (Vejchapipat et al, 2000), eventually resulting in failure of cellular homeostasis characterised by ion gradients across the cell membranes (McCord, 1987).

Although necrosis has long been considered the only mechanism leading to cell death in the ischaemic intestine (Park et al, 1990), recent evidence suggests that apoptosis also plays a major role in the destruction of intestinal epithelial cells (Ikeda et al, 1998). The pathogenic mechanisms leading to apoptotic cell death in this setting have not been fully elucidated, however it has been suggested that *anoikis* (disruption of epithelial cell-matrix interactions) may play an important part in induction of apoptosis in detached enterocytes.

1.9.2 Reperfusion

Restoration of blood circulation to ischaemic tissue is essential to re-establish oxygen and nutrient supply, and to remove catabolites generated during the ischaemic period, therefore preventing the progression of cell injury. However, reperfusion is associated with a series of physiological processes which worsen the initial injury established during ischaemia. Experimental studies have shown that tissue injury, particularly in the mucosa, is more severe following reperfusion of the ischaemic gut than after a comparable time of pure ischaemia (Parks et al, 1986). The pathogenesis of reperfusion injury is complex, and involves activation of several pathways often closely interconnected, including production of free radicals, release of cytokines and other soluble inflammatory mediators, recruitment of inflammatory cells, systemic diffusion of bacteria or their products, activation of the complement system, production of vasoactive substances and others.

Oxidative and nitrosative stress

Oxidative and nitrosative stress represent the harmful effects of free radicals causing potential biological damage. They are caused by an imbalance between the production of reactive oxygen (ROS) and nitrogen (RNS) species and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. The biologically reactive ROS are the superoxide anion (O_2^-), the perhydroxyl radical (protonated superoxide, HO_2^-), the hydroxyl radical (HO), and hydrogen peroxide (H_2O_2). RNS include free radical nitric oxide (NO) and peroxynitrite anion ($ONOO^-$), which forms from simultaneous

generation of NO and O_2^- (Beckman et al, 1990). ROS and RNS are highly unstable due to the presence of unpaired valence shell electrons, and reaction with biological compounds results in protein modification, lipid membrane disruption and deoxyribonucleic acid (DNA) damage.

ROS and RNS mediated reactions can cause structural alterations in DNA (e.g. nicking, base-pair mutations, rearrangements, deletions, insertions, and sequence amplification) (Cuzzocrea et al, 2001b). The hydroxyl radical HO is known to react with all components of the DNA molecule, damaging both the purine and the pyrimidine bases and also the deoxyribose backbone. NO or, more likely, reactive products derived from it, particularly peroxynitrite, are mutagenic agents, with the potential to produce nitration, nitrosation, and deamination reactions on DNA bases. Reactive species can also attack polyunsaturated fatty acid residues of phospholipids, which are extremely vulnerable to oxidation (Siems et al, 1995), initiating a series of chain reactions known as lipid peroxidation (see Figure 1.1). Once formed, peroxy radicals (ROO) can be rearranged via a cyclisation reaction to endoperoxides, with the final product of the peroxidation process being malondialdehyde (MDA) (Marnett, 1999). Changes in charge and physical structure of phospholipids in biological membranes can disrupt the integrity of the cell wall as well as that of other intra-cellular compartments, leading to cell death. The side chains of all amino acid residues of proteins, in particular cysteine and methionine, are susceptible to oxidation by the action of ROS and RNS (Stadtman, 2004). Oxidation of cysteine residues may lead to the reversible formation of mixed disulphides between protein thiol groups (-SH) and low molecular weight thiols, in particular glutathione (S-glutathiolation). Perhaps of more physiological importance, both ROS and RNS can affect signal transduction in post-hypoxic cells, and ROS are able to initiate cell death programs in the form of apoptosis or necrosis (Zhou et al, 2005; Irani, 2000; Semenza, 2000).

Oxidants are produced in excess during reoxygenation, but ROS and RNS production also may increase in the reduced state that characterises cellular hypoxia. The main sources of ROS during ischaemia and reperfusion include

xanthine dehydrogenase-oxidase, mitochondria, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.

Xanthine dehydrogenase-oxidase is a highly versatile enzyme which plays an important role in the catabolism of purines in mammals, and is particularly abundant in the liver and intestine (Krenitsky et al, 1974). Xanthine dehydrogenase catalyses the conversion of hypoxanthine to xanthine and uric acid using nicotinamide adenine dinucleotide (NAD^+) as an electron acceptor. Under normal conditions, the enzyme is mostly in the form of xanthine dehydrogenase, making this the preferred pathway for disposal of xanthine. During the ischaemic period, cellular ATP is catabolised to yield hypoxanthine, which accumulates in the cell. Prolonged hypoxia also triggers the conversion of xanthine dehydrogenase to the oxygen radical-producing xanthine oxidase, which uses molecular oxygen instead of NAD^+ as an electron acceptor (Parks et al, 1988). Superoxide anions are a by-product of this reaction and may be in turn converted to hydrogen peroxide and hydroxyl radicals (Granger et al, 1981). During reperfusion, molecular oxygen is reintroduced into the tissue, where it reacts with hypoxanthine and xanthine oxidase to produce a burst of oxygen free radicals. Although the mechanism of conversion of xanthine dehydrogenase to xanthine oxidase is not yet completely understood, it seems to depend on diffusion of Ca^{2+} into the cell due to ATP depletion, and subsequent cleavage of xanthine dehydrogenase to xanthine oxidase by a calcium-dependent protease (Harrison, 2002).

The mitochondrial respiratory chain is one of the most important sites of ROS production under physiological conditions, which is increased during cellular hypoxia and reoxygenation. The respiratory electron transport chain becomes reduced (i.e. the complexes harbour electrons) during anoxia, and the reduced state potentiates O_2 production. Mitochondria generate excess partially reduced oxygen species (primarily O_2^- and its dismutation product, H_2O_2) during reoxygenation, which react with respiratory chain proteins (especially those containing Fe-S centres) or diffuse as H_2O_2 out of mitochondria (Tong et al, 2000). Two sites in the respiratory chain, complex I and complex III, have been suggested to be the major ROS sources (Newmeyer et al, 2003). H_2O_2 produced by mitochondria might also

function as a signal transducer, allowing mitochondria to act as oxygen sensors that regulate extramitochondrial signalling pathways.

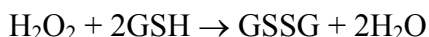
NADPH oxidase is an enzyme expressed by both phagocytic and non-phagocytic cell types, including enterocytes (El Hassani et al, 2005), and represents an important source of superoxide anions in physiological and pathological conditions. The classical phagocyte NADPH oxidase produces extracellular superoxide that plays a pivotal role in host defence against microbial infections. The non-phagocytic NADPH complex is structurally and functionally similar to phagocyte NADPH, resulting in reduction of molecular oxygen to generate superoxide (Babior, 2004). However, some features differentiate non-phagocyte from phagocyte NADPH. In particular, the non-phagocyte NADPH oxidases are constitutively active, producing relatively low levels of ROS under basal conditions and generating higher levels of oxidants in response to cytokines. Although the role of NADPH oxidase in reperfusion injury of the gut is still unclear, some evidence suggests a role for phagocytic NADPH oxidase in promoting leukocyte-endothelial interactions (Yusof et al, 2007; Korthuis et al, 1999).

Antioxidant defences against ROS and RNS include enzymatic systems, such as superoxide dismutase (SOD), glutathione peroxidase, and catalase, and non-enzymatic antioxidants, including ascorbic acid (vitamin C), α -tocopherol (vitamin E), glutathione, carotenoids, and flavonoids.

Glutathione is a tripeptide composed of three different amino acids: glutamate, cysteine and glycine. Two major redox forms of GSH have been identified in the cells, i.e. reduced glutathione (GSH) and glutathione disulphide (GSSG) (Figure 1.2). Glutathione redox state ($2\text{GSSH}/\text{GSSG}$) is a good indicator of the cellular redox state, and a measure of oxidative stress in a cell (Nogueira et al, 2004). The balance between reduced and oxidised glutathione is essential for cells survival, and elevated concentrations of oxidised glutathione may lead to oxidation and damage of enzymes. Glutathione is present at high concentrations in the cytosol (1-11mmol/l), nuclei (3-15mmol/l), and mitochondria (5-11mmol/l).

Glutathione exerts protection against oxidative stress through several different mechanisms (Masella et al, 2005). Firstly, it is a cofactor of several

detoxifying enzymes against oxidative stress, including glutathione peroxidase, glutathione transferase, and others. In particular, glutathione peroxidase reduces H_2O_2 (or other peroxides) to H_2O by oxidising glutathione as shown below:



or, in case of other peroxides:



Re-reduction of the oxidised form of GSSG is then catalysed by glutathione reductase through glutathione cycle:



An overview of the metabolic pathways involved in the reduction of free radicals by glutathione is illustrated in Figure 1.3.

The capacity to recycle GSH makes the GSH cycle pivotal to the antioxidant defence mechanism of a cell and prevents the depletion of cellular thiols.

In addition, glutathione can directly scavenge free radicals, including singlet oxygen and hydroxyl radical. Glutathione participates in amino acid transport through the plasma membrane. Finally, glutathione is able to regenerate the most important antioxidants, vitamin C and E, back to their active form. The potential of glutathione to regenerate the most important antioxidants is linked to the redox state of the glutathione disulphide-glutathione couple (Pastore et al, 2003).

The ratio of cytoplasmic GSH/GSSG has recently been implicated in a range of cellular processes, such as cellular signalling, gene expression and apoptosis (Rahman et al, 2005).

The normal GSH content of a cell is the function of the balance between its depletion and synthesis. Cells can either excrete GSSG or reduce it back to GSH in the cytoplasm by glutathione reductase as shown above. However, *de novo* synthesis of GSH from its amino acid constituents is essential for the elevation of GSH that occurs as an adaptive response to oxidative stress. GSH synthesis involves two enzymatic steps catalysed by glutamate cysteine ligase (the rate-limiting component) and glutathione synthetase (Huang et al, 1993). The *de novo* rate modulation of GSH synthesis also depends on the cellular levels of cysteine.

Cytokines

Cytokines are a group of small water-soluble proteins and glycoproteins that are involved in the regulation of both innate and adaptive immune responses. At low concentrations, cytokines can influence the cell from which they are produced and the surrounding cells in an autocrine and paracrine fashion. When released at higher concentrations, however, cytokines can enter the systemic circulation and influence the function of distant organs (endocrine effect). Cytokines have been traditionally divided into pro- and anti-inflammatory depending on their potential to stimulate or inhibit the inflammatory response. However, this classification appears artificial and simplistic: given the multiple effects of every cytokine, their impact on the inflammatory processes depends on the time and site of release, concentration, and on their interaction with the local conditions.

Traditional pro-inflammatory cytokines include interleukin-1 α , 1 β , 2, 6, 15 (IL-1 α , IL-1 β , IL-2, IL-6, IL-15), and TNF- α , whereas interleukin-4, 10, 12, and 14 (IL-4, IL-10, IL-12, IL-14), together with soluble IL-1ra are usually considered anti-inflammatory.

TNF- α is a powerful proinflammatory mediator that in many inflammatory responses, including I/R injury of many organs, serves as a central propagating factor (Frangogiannis et al, 2002; Donnahoo et al, 1999; Colletti et al, 1990; Seekamp et al, 1993). TNF- α promotes the inflammatory response primarily by stimulating signal transduction pathways leading to induction of gene expression for secondary inflammatory mediators. TNF- α activates a number of transcription factors regulating inflammatory gene expression including nuclear factor-kappa B (NF-kB), activator protein-1 (AP-1), as well as the p38 Map-kinase (MAPK). These transcription factors control gene expression for a variety of mediators including proinflammatory cytokines (i.e. IL-1, IL-6), chemokines (i.e. IL-8, and monocyte chemotactic protein 1 MCP-1), and vascular cell adhesion molecules (i.e. selectins, integrins).

As with many other acute inflammatory responses, TNF- α is a central mediator in the gut response to I/R. Increased levels of TNF- α have been demonstrated not only in the intestine (Wada et al, 2001; Souza et al, 2000), but

also in the systemic and portal circulation (Yamagishi et al, 2002; Squadrito et al, 1997; Fu et al, 1997) following mesenteric reperfusion. Mesenteric ischaemia and reperfusion injury has been shown to be substantially reduced by pharmacological inhibition of TNF- α (Esposito et al, 2007; Souza et al, 2007), and similar beneficial effects have been achieved using TNF- α receptor 1 knockout mice (Esposito et al, 2007; Cuzzocrea et al, 2004). This protection may be at least in part mediated via inhibition of NF-kB activation, which has been shown to prevent tissue injury and mortality following mesenteric reperfusion (Souza et al, 2005).

IL-6 is an acute phase cytokine that is produced by mononuclear phagocytes, fibroblasts, keratinocytes and endothelial cells and plays a central role in haematopoiesis, host defence and inflammation (Van, 1990). Clinical levels of IL-6 have been correlated with severity of disease in shock, trauma and transplanted organ rejection (Biffl et al, 1996b; Waage et al, 1989; Kita et al, 1994). IL-6 release can be induced by leukocytes in response to various stimuli, including LPS, heat shock response and IL-1. Although IL-6 is usually considered a pro-inflammatory cytokine, it also exhibits some anti-inflammatory properties, including down-regulation of cytokines such as IL-1 β , TNF- α and interferon-gamma (IFN- γ), and release of anti-inflammatory mediators such as IL-1ra and soluble TNF- α receptors (Opal et al, 2000). IL-6 levels in the systemic circulation remain unchanged during mesenteric ischaemia, but increase within minutes from the onset of reperfusion and remain elevated for several hours (Rocourt et al, 2007). The splanchnic origin of IL-6 has been suggested both by the increase in IL-6 messenger RNA (ribonucleic acid) in intestinal tissue, and by the higher levels of the cytokine in the portal and hepatic veins compared to arterial blood.

IL-10 is a pleiotropic cytokine and its main function is to limit and terminate inflammatory responses. IL-10 inhibits the activation and effector functions of T-cells, monocytes, and macrophages. It has effects on most haematopoietic cells and inhibits chemokine synthesis, nitric oxide production, expression of class II major histocompatibility complex (MHC) molecules, and proinflammatory cytokines (including TNF- α , IL-1 β , IL-6, IL-8, IL-12). IL-10 also regulates the growth and differentiation of B-cells, natural killer cells, and cytotoxic and helper T-cells. Effects of IL-10 are mediated both via the JAK/STAT

(Janus kinase/ signal transducer and activator of transcription) pathway, and through inhibition of NF- κ B activation (Boehler, 2002). IL-10 appears to play a crucial role in coordinating the anti-inflammatory gene response by modulating adhesion molecules and inflammatory cytokine gene expression in the early stages of reperfusion injury. However, the role of IL-10 is complex and experimental attempts at modulating IL-10 expression in animal models of mesenteric reperfusion have produced ambiguous results. IL-10 deficient mice showed a higher rate of mortality and more severe tissue injury following experimental mesenteric reperfusion compared with wild-type animals. This was mediated via an increase in proinflammatory cytokines TNF- α and IL-6, enhanced adhesion molecule expression such as P-selectin and intercellular adhesion molecule 1 (ICAM-1) and neutrophil infiltration, and development of oxidative and nitrosative damage (Zingarelli et al, 2001). Such protective effects have been confirmed by exogenously administered IL-10 both before, and 1 hour after the onset of reperfusion (Lane et al, 1997). However, other studies have suggested that both survival and intestinal injury are similar in wild-type and IL-10 deficient mice (Stallion et al, 2002), and that intravenous administration of IL-10 before reperfusion resulted in increased tissue damage, possibly via reduced inducible nitric oxide synthase (iNOS) expression (Nussler et al, 2003).

Inflammatory cells

Infiltration of neutrophils in tissues is believed to be responsible for important tissue damage not only at a local level (Sisley et al, 1994; Hernandez et al, 1987; Qing et al, 2001), but also in distant organs, such as lungs, liver and heart (Vinardi et al, 2003; Horton et al, 1993a; Horie et al, 1996; Simmons et al, 1990; Spasic et al, 1993) following intestinal I/R. In particular, neutrophil activation, neutrophil-endothelial adhesion and extravasation are fundamental steps in the pathophysiology of I/R injury.

Initial rolling of inflammatory cells along the endothelium is mediated by expression of adhesion molecules on the surface of endothelial cells, which is followed by firm adhesion and transendothelial migration (Ebnet et al, 1996). Evidence has been provided for the contribution of adhesion molecules to

leukocyte recruitment in several conditions (including localised inflammation, sepsis, and organ ischaemia and reperfusion) in response to the production of interleukins, TNF- α , LPS, histamine, PAF and other soluble mediators (Pettersen et al, 2002). Accumulation of activated neutrophils is driven by several chemoattractants, including C3a and C5a, IL-1 and TNF- α (Moraes et al, 2003), and it occurs in the intestinal, hepatic, and pulmonary vascular bed within minutes of intestinal reperfusion (Kurtel et al, 1991). Extravasated neutrophils degranulate and release a number of proteases from intracellular granules, including elastase, gelatinase, collagenase and cathepsin G. Neutrophil-derived proteases are able to directly cause cell death. In addition, mobilisation of azurophilic granules leads to the liberation of NADPH oxidase, which generates superoxide that dismutates to oxygen and hydrogen peroxide. Furthermore, myeloperoxidase released from the neutrophils azurophilic granules can generate hypochlorous acid (HOCl), another diffusible oxidant and chlorinating agent, which gives rise to other toxic species such as chloramines (Jaeschke, 2006).

Inhibition of neutrophil-mediated effects has been reported using neutrophil elastase inhibitors (Kotake et al, 2005; Takayama et al, 2001), blockade of adhesion molecules such as P-Selectin (Farmer et al, 2005; Carmody et al, 2004; Farmer et al, 2002) and ICAM-1 (Gayle et al, 2002; Kuzu et al, 2002), or chemokines (including cytokine-induced neutrophil chemoattractant or CINC) (Mbachu et al, 2004; Ishii et al, 2000). The fundamental role of neutrophils in contributing to mesenteric reperfusion injury is confirmed by the protective effects on local and distant organ damage of these strategies.

Recruitment of CD4⁺ and CD8⁺ T-lymphocytes mediated via endothelial adhesion molecule MAdCAM-1 (mucosal addressin cellular adhesion molecule 1) has also been proposed to contribute to intestinal reperfusion injury (Shigematsu et al, 2002). Mechanisms of T-cell dependent tissue injury include modulation of the neutrophil recruitment that occurs hours after reperfusion as well as the increased albumin extravasation that occurs within minutes after reperfusion.

In recent years, activated resident macrophages have been suggested to play a key role in the early phases of reperfusion-induced damage to the gut (Chen et al, 2004). I/R induced mucosal damage was correlated with activation of gut

resident macrophages and upregulation of myeloperoxidase and Early growth response factor 1 gene (EGR-1, a transcription factor involved in the inflammatory response). Depletion of gut resident macrophages by dichloromethylene bisphosphonate (Cl₂MBP) significantly reduced mucosal damage.

Adhesion molecules

The endothelium regulates immune and inflammatory responses by controlling the extravasation of leukocytes from the blood into tissues. Leukocytes enter inflamed tissue mostly through post-capillary venules that have been transiently activated by inflammatory stimuli. Activated endothelial cells express a complex series of molecules that is required to capture leukocytes and attract them into tissue. In particular, the activated endothelium expresses adhesive molecules that help leukocytes to engage the vessel wall in a protein-protein dependent interaction.

The initial interaction of leukocytes with the endothelium lining the vessel wall is known as tethering, and the subsequent rotational movement along the vessel wall is called rolling. These events are dependent on the selectins, a calcium-dependent, type I transmembrane glycoprotein family of adhesion molecules comprising E-selectin, P-selectin, and L-selectin, named for the cell type in which they were originally identified (endothelium, platelet, and leukocyte) (Kansas, 1996). P-selectin (CD62P, cluster of differentiation 62P) is constitutively expressed and stored in platelets α -granules and endothelial cells Weibel-Palade bodies. Within minutes from stimulation by inflammatory mediators (including oxidants, LPS, histamine, leukotrienes, or histamine), the Weibel-Palade bodies of endothelial cells fuse with the plasma membrane, increasing surface expression of P-selectin (Geng et al, 1990). This rapid expression accounts for the role of P-selectin in mediating early leukocyte recruitment during an inflammatory response. In addition, transcription of P-selectin can occur in rodents after activation of endothelial cells through expression of NF- κ B induced by inflammatory mediators such as TNF- α , IL-1, IL-4, and LPS (Pan et al, 1998).

E-selectin (CD62E, cluster of differentiation 62E) expression, on the contrary, requires de novo synthesis on endothelial cells, which is induced by

various mediators, such as IL-1, LPS, and TNF- α . Expression can occur on the endothelial surface as quickly as 2 hours after stimulation and generally decreases within 24 hours (Kansas, 1996). Despite delayed expression, E-selectin overlaps with P-selectin, helping to enhance leukocyte recruitment during inflammation. In fact, E-selectin expression results in a dramatic decrease in rolling velocity, therefore enhancing the probability of subsequent adhesion (Kunkel et al, 1996). L-Selectin (CD62L, cluster of differentiation 62L) is expressed on most leukocytes and mediates rolling in some inflammatory conditions, although its role appears secondary to the endothelial selectins (Jung et al, 1999).

Rolling is mediated by binding of selectins to specific ligands expressed by inflammatory cells, such as PSGL-1 (P-Selectin glycoprotein ligand 1). PSGL-1 is expressed on myeloid, lymphoid, and dendritic cells and can serve as a ligand to all selectins (Kansas, 1996). Leukocyte rolling on P-selectin is almost completely dependent on PSGL-1, and PSGL-1 is also an important ligand for E-selectin (Zanardo et al, 2004).

Following rolling, firm adhesion of leukocytes to endothelial cells is mediated by the interactions between integrins and their ligands on endothelial cells: ICAM-1, ICAM-2 and vascular cell adhesion molecule 1 (VCAM-1). Chemoattractant cytokines (or chemokines) are produced by activated leukocytes, macrophages, mast cells, and various tissue cells (including fibroblasts, epidermal cells, and endothelial cells) following release of pro-inflammatory cytokines such as IL-1, TNF- α , or IL-4 (Baggiolini et al, 1994). Chemokines bind specific receptors expressed on leukocytes to induce activation, including expression of integrins. Integrins consist of a heterodimer of an α and a β subunit, and are expressed by leukocytes and other cell types. Approximately 16 α subunits and 8 β subunits are known to exist, and various combinations form at least 22 heterodimers. Leukocyte adhesion to the endothelium is mediated by the β 2-integrins, fundamental for neutrophil infiltration (such as leukocyte function-associated molecule LFA-1, and macrophage 1 antigen Mac-1), as well as α 4-integrins, which are found primarily on monocytes, lymphocytes, and eosinophils (Luo et al, 2007). The ligands for leukocyte integrins are the immunoglobulin superfamily members expressed on endothelial cells, ICAM-1 and ICAM-2, and

VCAM-1, ICAM-1 and ICAM-2 are endothelial ligands for the leukocyte β 2-integrin LFA-1.

ICAM-1 is one of the most important adhesive receptors on endothelial cells for the firm adhesion of leukocytes, and can recognise several integrins including LFA-1 and Mac-1 (Springer, 1994). It is constitutively expressed on the endothelium, and it is further upregulated by inflammatory cytokines, including TNF- α , IL-1, and IL-4. In contrast, ICAM-2 is constitutively expressed and not inducible. ICAM-1 mediates firm attachment of leukocytes to the apical endothelial surface and the subsequent migration to endothelial cell contacts, a step called locomotion (Schenkel et al, 2004). Recent evidence suggests that ICAM-1 contributes to additional functions, including extravasation of leukocytes by diapedesis (transcellular migration) and signal transmission in endothelial cells (Yang et al, 2005; Tilghman et al, 2002).

Unlike ICAM-1, VCAM-1 is not constitutively expressed in most tissues but is strongly upregulated through de novo synthesis after stimulation with TNF- α and IL-1. VCAM-1 binds to both β 1 and α 4 integrins on monocytes, lymphocytes and neutrophils, and is mainly involved in the inflammation-related leukocyte migration although its blockade does not prevent leukocyte extravasation (Johnston et al, 2000).

Adhesion of leukocytes to endothelial cells is followed by leukocyte emigration. Leukocyte migration is still incompletely understood, although several proteins, including PECAM-1 (platelet endothelial cell adhesion molecule 1), CD99 (cluster of differentiation 99), junctional adhesion molecules, vascular endothelial-cadherins, and other molecules, have been implicated in this process (Engelhardt et al, 2004). Migration of adherent leukocytes into the extravascular space appears to take place both through intercellular junctions, and in a transcellular fashion through the endothelium (Shaw et al, 2001).

Bacterial translocation

Bacterial translocation is a phenomenon in which live bacteria or their products, including LPS and other toxins, cross the intestinal barrier. The human intestinal microflora contains 300 to 500 different species of bacteria, located

preferentially in the colon (Wiest et al, 2003). Although interaction between the host and its microbial guests determines important health benefits, some of these bacteria are potential pathogens and can be a source of local and systemic infection in case of failure of the mucosal barrier (Steinberg, 2003). The function of the barrier depends on the normal intestinal flora (ecological barrier), mucous epithelia (mechanical barrier), and secretory IgA and immune cells (immune barrier). Maintenance of the barrier relies on the integrity of cellular plasma membranes and tight junctions, as well as the elaboration of endothelial and epithelial secretory products (Baumgart et al, 2002).

Intestinal epithelial cell hypoxic injury and subsequent reperfusion induces profound disruption of the intestinal barrier function. Translocation might be mediated by a three-hit model as proposed by Deitch (Deitch, 2002). The first gut insult could be hypoperfusion and ischaemia. Acidosis and overproduction of reactive oxygen species determine disarrangement in the mucosal cytoskeleton, thus increasing epithelial permeability. Restoration of the intestinal blood flow produces a second hit, with migration of neutrophils to the intestinal microcirculation, release of cytokines by leukocytes and GALT (Gut-Associated Lymphoid Tissue), and generation of prostaglandins, thromboxane, prostacyclins and leukotrienes causing increased vascular permeability. The third hit is the loss of integrity of the gut barrier function, providing translocation of intestinal endotoxins and bacteria, and exposure to immune cells. The majority of these bacteria is phagocytosed and contributes to the intestinal inflammatory response. However, some translocated bacteria and toxic compounds are drained by the mesenteric lymphatic system and are trapped in the intestinal lymph nodes, causing an inflammatory reaction.

Current data suggest two major pathways of gastrointestinal permeability through which translocation may occur: transcellular through the enterocytes and paracellular using the tight junctions (Ellis, 2004). In the same way, there are two major routes that bacterial compounds might gain access to the systemic circulation: through the enteric venous system to the portal vein, or following lymphatic enteric drainage. Recent evidence suggests that the latter might be the principal pathway of translocation (Kaneko et al, 2007).

Evidence from experimental studies shows that LPS release into the circulation is dramatically increased following reperfusion of the ischaemic gut (Yamagishi et al, 2002; Ohara et al, 2001). Following prolonged intestinal reperfusion, bacterial colonization has been documented not only in mesenteric lymph nodes, but also in distant organs such as liver and spleen (Fu et al, 1997; Yao et al, 1995; Ohara et al, 2001; Kucukaydin et al, 2000). The importance of bacteria and their products in contributing to reperfusion injury is confirmed by the reduction of local and distant organ injury via modulation of the systemic inflammatory response achieved via gut decontamination or germ-free environment (Sorkine et al, 1997; Souza et al, 2004).

Complement and natural antibodies

The complement system consists of over 20 small proteins and protein fragments normally circulating as inactive zymogens in the blood. Upon activation by one of several triggers through the classical, alternative, or mannose-binding-lectin associated pathway, proteases in the system cleave specific proteins to release cytokines and initiate an amplifying cascade of further cleavages. This activation cascade finally results in release of opsonising (C3b, C4b, and iC3b) and chemotactic (C5a) molecules, as well as in direct cytolytic effect (via formation of the membrane attack complex) and activation of the inflammatory cascade through generation of anaphylatoxins (C3a and C5a) (Cole et al, 2003). The activation of the complement cascade following intestinal I/R may depend on both the classical (Woodcock et al, 2000) or the alternative pathway (Stahl et al, 2003).

It has recently been suggested that the importance of the complement system in the pathogenesis of tissue injury is mediated via circulating natural antibodies as part of the innate immune response. Innate immune B cells are T cell-independent B cells present in the primary, pre-antigen-driven repertoire. These innate immune B cells participate in the host response against microbial antigens through the generation of natural IgM (immunoglobulin M) antibodies that do not require affinity maturation to provide early protection (Milner et al, 2005). Although beneficial for the host, these cells have also been implicated in the pathogenesis of autoreactivity. Recent evidence proves that circulating natural IgM

contribute to the pathogenesis of reperfusion injury of several organs, including the intestine, by binding to specific self-antigens exposed during ischaemia and reperfusion. It has been suggested that brief periods of ischaemia lead to an alteration in surface epitopes of self-antigens. Natural IgM would recognize these neo-antigens on damaged tissue and, upon binding to the cell surface, initiate inflammation by activating complement in the classical pathway (Williams et al, 1999). Recent experiments have shown that a specific IgM isolated from a single hybridoma clone was sufficient to restore reperfusion injury in a mouse model (Zhang et al, 2004). This effect was highly specific, because IgM prepared from 20 other hybridomas did not restore injury. Non-muscle myosin heavy chain type II A and C, a highly conserved self-antigen, has been shown to be the target of natural IgM that can initiate intestinal I/R injury (Zhang et al, 2006), although phospholipids may represent other possible target antigens (Fleming et al, 2004).

Nitric oxide

Nitric oxide (NO) is a free radical and a highly reactive substance. NO is synthesised by a family of enzymes known as NO synthases (NOS) utilising *L*-arginine as a precursor. Three isoforms of NOS have been identified and cloned: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Albrecht et al, 2003). eNOS is expressed at low concentrations in the mesenteric endothelium; under physiological conditions, it regulates intestinal blood flow by acting as a vasodilator, and its inhibition results in an increased splanchnic vascular resistance (Pique et al, 1992). nNOS is the most abundant isoform in the gut, and is expressed in the myenteric plexus (Hoffman et al, 1997). Unlike eNOS and nNOS, which are constitutively expressed, iNOS is virtually absent in the intestinal mucosa under normal conditions, and is expressed by leukocytes and other cell types in response to inflammatory stimuli (e.g. cytokines) and growth factors.

The role of NO in hypoxia and reoxygenation is multifaceted, and still largely unclear. On one hand, NO is a cytoprotective agent, and inhibition of NO production causes tissue dysfunction in certain models of mesenteric reperfusion. However, NO can act as a cytotoxic compound and its blockade can provide

beneficial effects. Although apparently paradoxical, these different effects can be at least in part explained by the different sites and sources of NO production, as well as by the different regulatory mechanisms.

Intestinal I/R is associated with impaired endothelial function, which would result in an overall reduced production in NO (Grace, 1994). Administration of *L*-arginine, the precursor for NO synthesis, has been shown to protect from mesenteric reperfusion injury in several studies (Kozlov et al, 2002; Ward et al, 2000; Horie et al, 1997b; Schleiffer et al, 1996), although this is controversial (Fukatsu et al, 2004).

A more clear understanding of the pathophysiology of NO has been provided by studies utilising selective inhibitors of the different isoforms of NOS. The constitutive isoforms of NOS (nNOS and eNOS) are critical to normal physiology, and selective inhibition of these enzymes has consistently been shown to be detrimental (Kubes et al, 2000). The induction of iNOS produces large amounts of NO, which leads to increased mucosal permeability, causes mucosal injury, promotes bacterial translocation, and decreases mesenteric blood flow (Suzuki et al, 2000). Consistent with these findings, specific inhibition of this enzyme has shown to reduce local and distant organ injury following mesenteric reperfusion (Naito et al, 2004; Wu et al, 2002; Chen et al, 2000; Barocelli et al, 2006; Uchida et al, 2007). Although the molecular bases for the deleterious effects of iNOS-derived NO have not been completely elucidated, the generation of peroxynitrite from the contemporary release of high amounts of superoxide and NO plays a crucial role (Cuzzocrea et al, 2001b).

Endothelins

Endothelins are potent vasoconstrictors released by the vascular endothelium (Ozel et al, 2001). Three active isoforms of endothelin (ET-1, ET-2, and ET-3) have been described. Their vasoconstrictive effects are predominantly mediated via ET-A receptors present on smooth muscle cells of blood vessels via release of calcium ions from intracellular stores. Interaction of endothelins with ET-B receptors mediates either vasoconstriction (ET-B2), or vasodilatation (ET-B1). Intestinal I/R induces an increase in endothelin concentrations in the portal

vein, which may exacerbate reperfusion injury (Schlichting et al, 1995), whereas inhibition of endothelins by receptor blockade protects against reperfusion injury of the gut (Wolfard et al, 2002; Oktar et al, 2002; Wolfard et al, 1999).

1.10 MULTIPLE ORGAN DYSFUNCTION SYNDROME

MODS, also known as multiple organ failure, is characterised by multiple and progressive organ dysfunction severe enough to require intervention for homeostasis in an acutely ill patient. MODS is one of the leading causes of mortality in surgical intensive care units, and accounts for the majority of deaths in clinical conditions associated with intestinal I/R in both adults (Sauaia et al, 1995; Sharma et al, 2003) and paediatric patients (Carcillo, 2003).

The pathogenesis of MODS following reperfusion injury of the gut, similarly to that of other organs, relies on the development of a systemic inflammatory response mediated by ROS and RNS, cytokines, activated complement, eicosanoids, and leukocytes as described before. The complete disruption of the intestinal mucosal barrier secondary to the ischaemic insult determines massive translocation of bacteria and endotoxins from the intestinal lumen into the circulation (Deitch, 1990), unlike reperfusion injury in cardiac, cerebral, and hepatic disease where this phenomenon is more limited. Once initiated, the systemic inflammatory response instigates a self-enhancing vicious circle leading to a progressively more severe injury not only to the intestine, but also to distant organs such as the lungs, heart, liver, kidneys, and the microcirculation (Stallion et al, 2005).

1.10.1 Lungs

The lung is the most commonly affected vital organ in critically ill patients, and acute respiratory distress syndrome (ARDS) usually precedes failure of other organs in patients with MODS (Moore et al, 1996). Respiratory failure is characterised by pulmonary oedema due to enhanced microvascular permeability and increased hydrostatic pressure through the pulmonary capillary bed (Iglesias et al, 1998). Using *in vivo* confocal laser microscopy, macromolecular leak in the

interstitial space and the alveoli was observed both during ischaemia and, to a much greater extent, upon reperfusion of the intestine. In addition, capillary obstruction by stagnating leukocytes or micro-thrombi has been described in the pulmonary vasculature as a consequence of mesenteric reperfusion (Mitsuoka et al, 1999). This pulmonary microvascular injury leads to altered gas exchange with altered excretion of carbon dioxide and impaired oxygenation, and subsequent hypoxemia and hypercapnia (Nuckton et al, 2002).

Inflammatory mediators such as PAF, TNF- α and other cytokines, eicosanoids, activated complement proteins, chemokines, and iNOS derived NO have been shown to contribute to the pathogenesis of pulmonary oedema following mesenteric reperfusion (Kostopanagiotou et al, 2007; Yang et al, 2007; Akahori et al, 2006; Xiao et al, 1997; Uchida et al, 2007). In addition to soluble mediators, neutrophils are believed to play a key role in lung injury, and inhibition of neutrophil functions (Kotake et al, 2005) or recruitment (Kuzu et al, 2002) effectively reduces intestinal reperfusion-induced pulmonary injury.

1.10.2 Heart

Cardiac function has been shown to be profoundly affected following experimental intestinal I/R, with a progressive impairment in myocardial function, which could exacerbate existing intestinal hypoperfusion (Horton et al, 1991; Horton et al, 1993a). Intestinal reperfusion injury causes both histological injury and functional impairment. Histological damage following mesenteric reperfusion is characterised by myocardial interstitial oedema, neutrophil infiltration, and myocyte necrosis (Yao et al, 1996; Douzinas et al, 2003). Functional impairment in both myocardial contraction and relaxation has been shown to develop as early as 2 hours after the onset of intestinal reperfusion, which persists for up to 16 hours in isolated perfused hearts (Horton et al, 1991).

Such myocardial dysfunction and injury seems to be mediated via cytokine release, free radicals production, and neutrophil activation, since inhibition of these pathways by administration of TNF- α inhibitors, free radical scavengers (superoxide dismutase, catalase, allopurinol) or pentoxifylline (inhibitor of

neutrophil adherence and aggregation) prevents cardiac dysfunction (Yao et al, 1996; Horton et al, 1993a; Horton et al, 1993b; Horton et al, 1991).

1.10.3 Liver

As previously discussed, venous outflow from the mesenteric circulation is filtered through the liver via the portal circulation before entering the inferior vena cava, which makes the hepatic parenchyma particularly vulnerable to the consequences of intestinal reperfusion injury. Firstly, a reduction in portal blood flow to a third of baseline is observed during mesenteric ischaemia (Turnage et al, 1996). Upon intestinal reperfusion, recovery of hepatic inflow is only partial and does not exceed 85% of baseline levels (Nakamura et al, 2001), suggesting the presence of alternative mechanisms other than a reduced portal venous return. In fact, a substantial reduction in blood flow via the hepatic artery (Turnage et al, 1996) and reduced sinusoidal perfusion have been documented upon experimental mesenteric reperfusion (Horie et al, 1996; Yamagishi et al, 2002).

Hypoperfusion is accompanied by liver tissue ischaemia, which is worsened during mesenteric reperfusion: in rabbits subjected to mesenteric artery occlusion-revascularisation, tissue oxygenation index drops to 50% of baseline after 90min intestinal ischaemia, and continues to decrease after restoration of mesenteric blood flow (Horie et al, 1997a). Histological changes as documented by light and electron microscopy include progressive hepatocyte damage with cytoplasm oedema and condensation of the nucleus, tissue oedema and haemorrhage, leukocyte infiltration, and blood cell aggregates in the sinusoidal spaces (Tian et al, 2006; Giakoustidis et al, 2006).

Using *in vivo* magnetic resonance spectroscopy, Vejchapipat et al demonstrated that significant liver energy failure is observed upon reperfusion, but not during mesenteric ischaemia alone (Vejchapipat et al, 2001). This failure is characterised by a 50% decrease in hepatic ATP during mesenteric reperfusion, which is the fundamental source of energy for cellular activities, together with an increase in end-products of anaerobic metabolism such as succinate and lactate. Interestingly, this drop in hepatic ATP levels immediately preceded death of

experimental animals, suggesting a possible association between hepatic energy failure and mortality.

Although the exact pathogenesis of such hepatic injury remain unclear, several possible mechanisms have been proposed, including bacterial translocation and release of cytokines (such as IL-1, IL-6 and TNF- α) in the portal circulation (Medeiros et al, 2006; Kaplan et al, 2007). In addition, sequestration of neutrophils in hepatic sinusoids with plugging by leukocyte and leukocyte-platelets aggregates has been shown to contribute to hepatic injury (Horie et al, 1997a). This appears to be mediated via expression of endothelial adhesion molecules such as ICAM-1 and P-selectin, possibly through up-regulation of transcription factor NF-kB (Tian et al, 2006; Yamagishi et al, 2002).

1.10.4 Kidneys

Although renal failure is common in the clinical setting of mesenteric reperfusion injury, few experimental studies focused on the consequences of intestinal I/R on the kidneys. Renal blood flow has been shown to be reduced to 20% of baseline in rats after 60min of mesenteric reperfusion (LaNoue et al, 1996). Renal hypoperfusion was accompanied by tissue hypoxia (as confirmed by a 25% reduction in cellular ATP levels) and renal tubular dysfunction. Electronic microscopy studies revealed the presence of structural alterations of both the endothelial cells of the glomerular capillaries, and epithelial cells of the tubules in the early phases of mesenteric reperfusion, possibly as a consequence of lipid peroxidation in the kidney (Mutlu et al, 2002; Mura et al, 2007). Increased release of reactive oxygen species, imbalance in eicosanoid production, and neutrophil-induced tissue injury have been proposed as possible mechanisms in the pathogenesis of renal dysfunction, as confirmed by the protective effects of allopurinol and pentoxifylline in experimental models (Rothenbach et al, 1997).

1.11 HYPOTHERMIA IN INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

Therapeutic hypothermia consists in a controlled lowering of core body temperature below normal values (<35°C), and can be classified as mild (32 - 34

°C), moderate (28 - 32 °C), or deep (<28 °C). Previous studies in rats have suggested that application of moderate hypothermia throughout ischaemia and reperfusion of the gut can substantially prevent mortality (Vejchapipat et al, 2001), and these effects have been shown to persist after rewarming of the experimental animals (Kalia et al, 2002b). Unlike most other therapeutic strategies applied during ischaemia and reperfusion injury, hypothermia does not act on one single target, but it rather modulates several different pathways involved in the development of both local and distant organ damage, including modulation of metabolism, of the inflammatory response, and of heat shock response.

The metabolic requirement for oxygen and energetic substrates of the small intestine is high because of its absorptive and secretory functions, which is not affected by a reduction of body temperature to 32°C in otherwise healthy animals. Although whole-body moderate hypothermia has shown to reduce mesenteric blood flow in newborn and neonatal piglets (Powell et al, 1999), the oxygen delivery to those organs is likely to be enough to meet the decreased metabolic demand of splanchnic viscera. This is confirmed by the fact that intestinal and hepatic phosphoenergetics were unaffected after 120min of moderate hypothermia in sham-operated rats (Vejchapipat et al, 2006b). However, interruption of blood supply during ischaemia causes a rapid and dramatic decrease in intestinal concentrations of ATP and phosphocreatine in normothermic rats, which drops to 20% of baseline within 10min from the onset of mesenteric ischaemia (Blum et al, 1986). Interestingly, restoration of blood supply fully reverts this energetic failure only for short ischaemia times, whereas a permanent time-dependent decrease in the recovery of high energy phosphoenergetics is observed if the duration of ischaemia is superior to 10min. These data suggest that if the initial energy failure may be simply due to a lack of oxygen and energetic substrates, and therefore reversible upon restoration of perfusion, long periods of hypoperfusion result in irreversible derangement of energy metabolism.

These results have been confirmed in a similar animal model where longer ischaemia and reperfusion times were studied (Vejchapipat et al, 2000b; Vejchapipat et al, 2001; Vejchapipat et al, 2002). During prolonged intestinal ischaemia (90min and 150min), phosphocreatine, ATP, adenosine di-phosphate

(ADP), and glucose levels in the ileum dropped substantially, whereas inorganic phosphate, succinate, and lactate level markedly increased. This reflects the impairment of oxidative phosphorylation, with accumulation of tricarboxylic acid cycle intermediates and by-products of anaerobic glycolysis. Prolonged ischaemia followed by 60min of intestinal reperfusion was not followed by resynthesis of phosphoenergetics in intestinal tissue despite partial recovery of glucose, succinate, and lactate, suggesting the presence of severe intestinal damage as indicated by irreversible intestinal energy failure.

Application of whole-body moderate hypothermia (30 to 32°C) throughout both the ischaemic and the reperfusion phase resulted in partial recovery of intestinal phosphocreatine, ATP, and lactate levels only when a model of 30min of ischaemia and 60min of reperfusion was used, whereas for 90min of ischaemia followed by 60min of reperfusion no hypothermic protection was observed. Interestingly, the preservation of energy metabolism by hypothermia was associated with an improvement in reperfusion-induced histological injury in the terminal ileum. The beneficial effects of moderate hypothermia on intestinal energy metabolism are therefore likely to be ischaemic-time dependent, but the mechanism of hypothermic protection is still unclear. Although the temperature-dependent reduction in metabolic rate is a well-known phenomenon, the small decrease in oxygen consumption caused by moderate hypothermia cannot explain the ability to preserve the viability of tissue, as the oxygen reserves would still be rapidly consumed in the ischaemic state (Kataoka et al, 1998).

Total-body hypothermia also induced protection from liver energy failure in the same animal model, which was associated with improved survival (Vejchapipat et al, 2001). In particular, normothermic reperfusion was associated with progressive liver energy failure, as indicated by a decrease in ATP together with an increase in hepatic inorganic phosphate to ATP ratio. Induction of moderate hypothermia throughout the experiment delayed the onset of liver energy failure and prevented mortality. Although it is unclear whether this beneficial effect is due to primary protection of the intestine, or to a direct effect on the liver, hepatic down-regulation of pro-inflammatory and pro-apoptotic signal transducers

and activators of transcription (STATs) may be involved in hypothermic protection (Parkinson et al, 2004).

Modulation of the inflammatory response induced by cooling also seems to play a crucial role in hypothermic protection. Hypothermia has been reported to delay production of cytokines (such as IL-1 β , TNF- α , IL-6) in human polymorphonuclear and mononuclear cells in vitro (Fairchild et al, 2000; Kimura et al, 2002), and to reduce production of IL-1 β in human cerebrospinal fluid “in vivo” (Marion et al, 1997). In addition, moderate hypothermia inhibits human monocytes activities, including chemotaxis, phagocytosis and bacterial killing (Kimura et al, 2002). Kalia et al investigated leukocyte adhesion to the villus endothelium in rats undergoing 30min superior mesenteric artery occlusion followed by 120min reperfusion by utilising in-vivo fluorescence microscopy and acridine orange labelled leukocytes (Kalia et al, 2002b). As expected, a rapid, sustained, and significant increase in leukocyte adherence within both the mucosal capillaries and the supplying arteriole occurred upon mesenteric reperfusion in normothermic animals. However, in animals maintained at moderate hypothermia throughout mesenteric ischaemia and reperfusion, leukocyte adhesion to the endothelium was not only delayed but also of significantly lower magnitude, and did not increase following rewarming of hypothermic animals. This effect may be explained by the potential of hypothermia to reduce adhesion molecule-mediated leukocyte-endothelial interactions, as proved by the reduced levels of soluble ICAM-1 (sICAM-1) by hypothermia in a similar animal model (Vejchapipat et al, 2006a). Down-regulation of pro-inflammatory transcription factor NF-kB and of iNOS in the intestine by hypothermia have also been reported in different animal models where selective hypothermia of the gut was used, which would contribute to reduce the extent of the inflammatory response (Montero et al, 2003; Attuwaybi et al, 2003). Interestingly, further experiments in rats showed that the anti-inflammatory effects of cooling may not be limited to the gut, as suggested by the reduction in neutrophil infiltration in the lung following mesenteric reperfusion (Vinardi et al, 2003).

1.12 CLINICAL APPLICATIONS OF HYPOTHERMIA

The main clinical indications for whole-body therapeutic hypothermia include cardiac arrest, neonatal hypoxic ischaemic encephalopathy, traumatic brain injury, surgical procedures with cardiopulmonary bypass, and acute liver failure.

Several clinical studies have suggested that mild induced hypothermia may improve neurologic outcome in survivors from cardiac arrest (Cheung et al, 2006). Cardiac arrest causes an immediate cessation of blood flow, leading to a rapid depletion of cerebral oxygen and ATP stores, and depressed cerebral function. There are three phases of cerebral injury after hypoxic insult: early, intermediate and late. Therapeutic hypothermia is considered to be neuroprotective by acting at each of the 3 stages of injury, perhaps synergistically (Green et al, 2005). Immediately after arrest of cardiac activity, cerebral blood flow decreases significantly despite ongoing consumption of oxygen and energetic substrates. Application of hypothermia in this early stage can improve energy metabolism by reducing consumption of oxygen and glucose. The intermediate or latent phase occurs in the hours post-arrest, which is characterised by the activation of cytotoxic cascades in the brain, including release of free radicals and nitric oxide. Hypothermia decreases the release of excitatory amino acids and other neurotoxic mediators, and reduces nitric oxide production. The late phase of cerebral injury can occur up to 24 hours following cardiac arrest. This stage is characterised by a break-down in the blood-brain barrier, development of progressive cerebral oedema, and eventually neuronal death. Application of hypothermia defers the deterioration of the blood-brain barrier and decreases cerebral oedema (Howes et al, 2006). A recent meta-analysis investigated the outcome of 385 survivors of cardiac arrest that were randomised to standard or mild hypothermia therapy (core body temperature 32-34°C) for 24 hours, when treatment was applied within 6 hours after arrival at the emergency department (Holzer et al, 2005). The results showed that hypothermia substantially increased the chance of survival with favourable neurological outcome at discharge from hospital (risk ratio, 1.68; 95% confidence interval, 1.29-2.07), with a number-needed-to treat of 6 (95% confidence interval, 4-13). A similar protection from hypothermia was observed in

the 6 month follow-up. Although potential complications from patients cooling have been reported, including increased infection risk, coagulopathy, and arrhythmias with depressed cardiac function (Cheung et al, 2006), only a trend towards higher incidence of bleeding or sepsis was seen.

Therapeutic hypothermia has also been widely applied following peripartum asphyxia to prevent hypoxic ischaemic encephalopathy in newborn infants. Magnetic resonance spectroscopy studies in term infants with evidence of intrapartum hypoxia have shown that neuronal death follows a “biphasic” model. Cerebral oxidative metabolism appears to be normal shortly after birth followed by secondary energy failure, the degree of which predicts both mortality and long-term neurodevelopmental outcome (Roth et al, 1997). Therefore, a therapeutic window of opportunity exists in the interval following resuscitation of the asphyxiated newborn before the secondary phase of impaired energy metabolism and injury occurs. Experimental studies have shown that hypothermia may confer neuroprotection by a number of different mechanisms, including reducing cerebral metabolic rate, blunting the inflammatory response, lowering production of reactive oxygen and nitrogen species, and down-regulating apoptotic cell death (Shankaran et al, 2007).

Clinically, hypothermia can be induced by two different methods: whole body cooling and selective head cooling with mild systemic hypothermia. Although selective cooling of the brain offers the theoretical advantage of minimising the adverse effects of systemic cooling, it has been shown that reducing the temperature of the whole body is essential in order to achieve a significant reduction in deep brain temperature (Van Leeuwen et al, 2000). A recent meta-analysis reviewed the outcome of 8 randomised controlled trials including 683 near-term infants with moderate or severe encephalopathy and evidence of intrapartum asphyxia (Jacobs et al, 2007). Therapeutic hypothermia resulted in a clinically important reduction in the combined outcome of mortality or long-term major neurodevelopmental disability (risk ratio, 0.76; 95% confidence interval 0.65-0.89), with a number needed to treat of 7 (95% confidence interval 4-14). Cooling also resulted in statistically significant reductions in mortality and neurodevelopmental disability in survivors.

Interestingly, these benefits were more pronounced in newborns with severe encephalopathy compared to those with encephalopathy of moderate degree. Reported adverse effects of hypothermia included increased thrombocytopenia, and hypotension requiring inotropic support.

Whole-body hypothermia has also been extensively applied in both adult and paediatric patients with traumatic brain injury. However, three meta-analyses reported conflicting results with regard to the potential of cooling to reduce the risk of mortality and adverse neurological outcome (Alderson et al, 2004; McIntyre et al, 2003; Henderson et al, 2003), and hypothermia is not currently recommended as a standard of care (Shafi et al, 2006).

Total body cooling has also been applied for over 50 years in patients undergoing coronary artery bypass surgery in order to reduce neurological damage, although its efficacy remains controversial. A meta-analysis of 19 randomised controlled trials failed to demonstrate a clear advantage of hypothermic versus normothermic cardiopulmonary bypass (Rees et al, 2001). Although patients in the hypothermia groups showed a reduced stroke rate, this is offset by an increase in non stroke related perioperative mortality and myocardial damage.

Therapeutic hypothermia has also been described in patients with severe acute liver failure in an attempt to control intracranial hypertension refractory to mannitol (Jalan et al, 2004), and to stabilize intracranial pressure during orthotopic liver transplant (Jalan et al, 2003), although further studies are needed before this therapy is recommended.

1.13 AIMS OF THE THESIS

The aims of this thesis are:

1. To study the consequences of intestinal reperfusion injury on the inflammatory response, oxidative and nitrosative stress, and energy metabolism in the gut and distant organs in experimental models of adult and infant intestinal I/R.

As discussed previously in this Chapter, there are significant differences between adult and newborns in the susceptibility to ischaemic injury, as well as in the pathophysiology of the body's response to mesenteric I/R. In addition, there is very little experimental evidence elucidating the pathogenesis of MODS in the newborn period. I therefore designed a series of experiments in both adult and developing rats, using a model of mesenteric artery occlusion and deocclusion, in order to investigate the potential pathways responsible for both intestinal injury and distant organ failure in human diseases associated with intestinal I/R injury.

2. To evaluate the effects of moderate hypothermia as a potential novel therapy for intestinal I/R injury in adult and developing experimental animal models.

The effects of hypothermia were investigated both when applied as a preventative therapy throughout the ischaemic and reperfusion phase, and when applied as a rescue therapy after the onset of reperfusion. As discussed in the previous paragraph, the potentials of moderate hypothermia as a therapeutic measure in this setting are extremely promising due to both its profound effects on multiple different pathways, and to the high safety profile and limited side effects shown in numerous neonatal trials.

3. To investigate the potential benefits of 5,10,15,20-tetrakis(*N*-methyl-4'-pyridyl)porphyrinato iron (III) (FeTMPyP) in an infant model of intestinal I/R injury.

FeTMPyP is a recently developed, selective antioxidant acting as a peroxynitrite decomposition catalyst. As discussed previously in this Chapter, selective scavenging of peroxynitrite offers some theoretical advantages over older, less selective antioxidants such as inhibitors of superoxide dismutase (SOD), by simply blocking the deleterious effects of this "ugly" free radical without interfering with the potentially beneficial

functions of other radicals (eg bacterial killing, intracellular signalling and others) (Beckman et al, 1996).

4. To further elucidate the pathogenesis of human NEC, with particular focus on characterising the inflammatory process in the intestine and evaluating its relationship with clinical outcome.

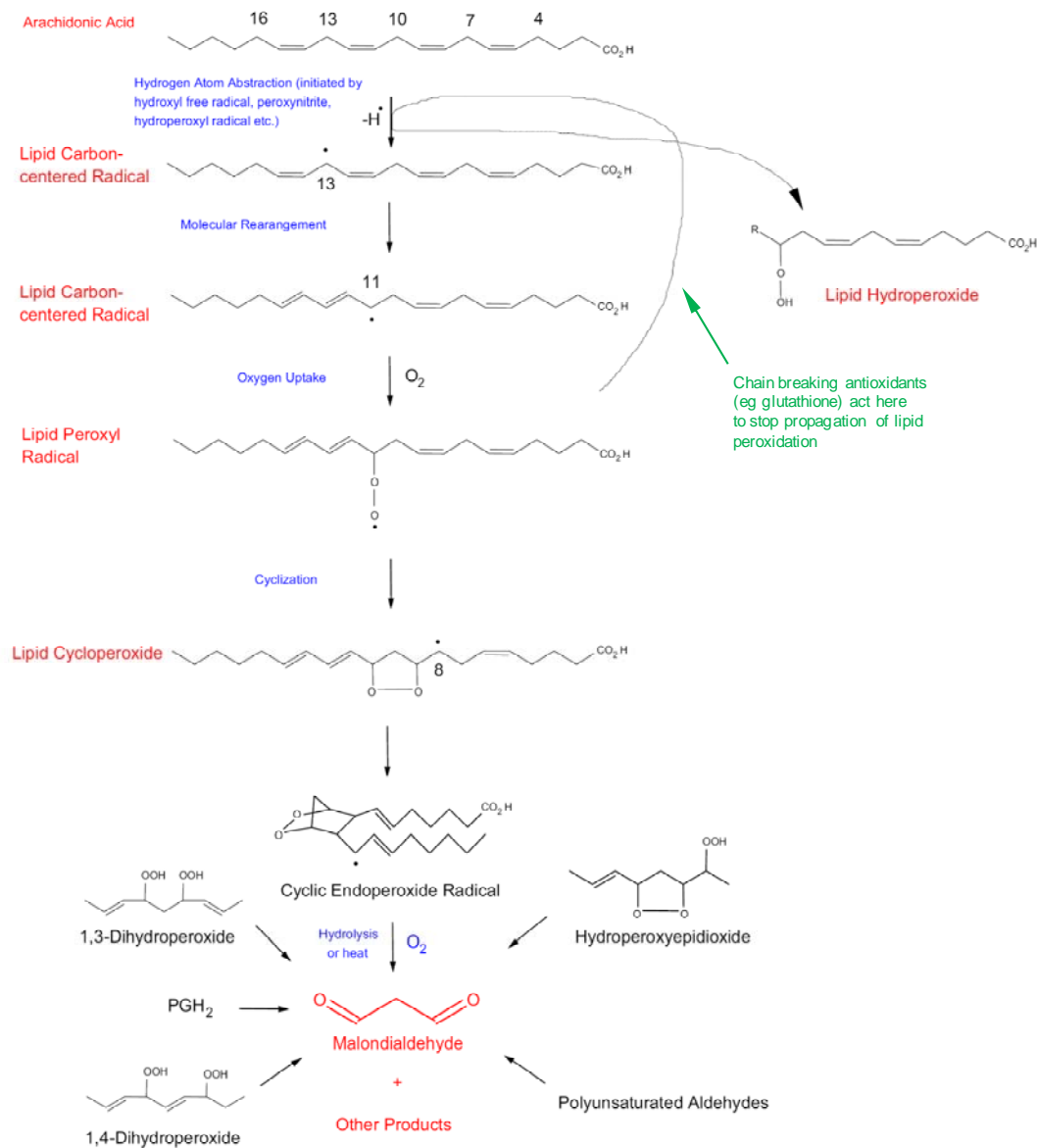
Although the clinical features of human NEC have been extensively described in the literature, the characteristics and role of the inflammatory response in the intestine are largely unknown. Contributing to fill this gap is particularly important not only to offer a better understanding of the pathogenesis of the disease, but also in order to compare the findings of my experimental studies in rat models of intestinal I/R with a human condition where investigational therapies could be applied. In particular, establishing a parallel between the inflammatory response in human disease and experimental conditions would reinforce the potential clinical benefits of a thereapeutic strategy that proved to modulate it in an animal model.

Table 1.1. Modified Bell staging for necrotizing enterocolitis (Walsh et al, 1986)

Stage	Systemic signs	Abdominal signs	Radiographic signs
I Suspected NEC	Temperature instability, apnoea, bradycardia, lethargy	Gastric retention, abdominal distension, blood in the stools	Normal or intestinal dilation, mild ileus
IIA Mild NEC	Same as above	Same as above, plus marked abdominal distension ± tenderness, absent bowel sounds, grossly bloody stools	Intestinal dilation, ileus, focal pneumatosis intestinalis
IIB Moderate NEC	Same as above, plus mild metabolic acidosis and thrombocytopenia	Same as above, plus abdominal wall oedema and tenderness ± palpable mass	Same as IIA, plus extensive pneumatosis, early ascites ± portal vein gas
IIIA Severe NEC	Same as IIB, plus hypotension, oliguria, mechanical ventilation, combined respiratory and metabolic acidosis, DIC	Same as above, plus worsening wall oedema with erythema and induration	Same as IIA, plus prominent ascites, fixed bowel loop
IIIB Severe NEC	Same as IIIA, further deterioration and shock	Evidence of perforation	Same as above, plus pneumoperitoneum

DIC: disseminated intravascular coagulation

Figure 1.1. Lipid peroxidation and formation of malondialdehyde.

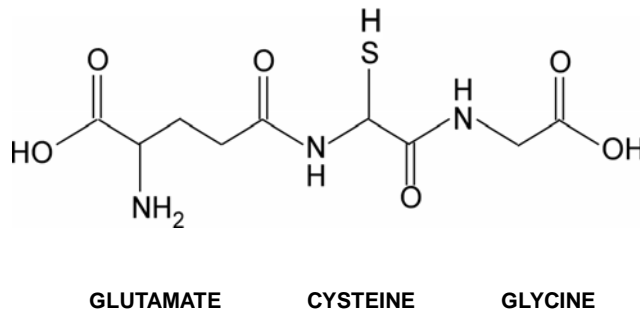


Lipid peroxidation of a poly-unsaturated fatty acid leads to the formation of malondialdehyde as end-product.

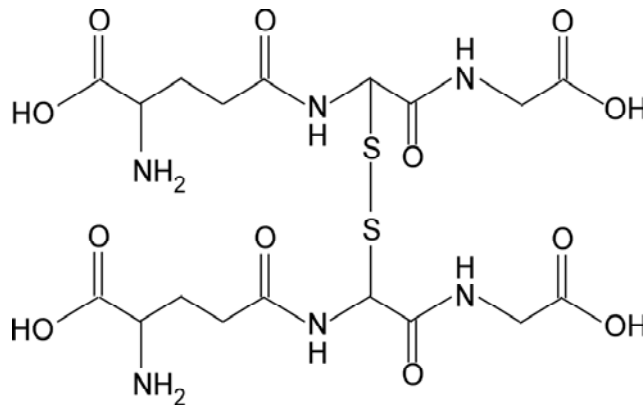
(Modified from ESA Application note 70-5033, Thermo fisher Scientific, Chelmsford, MA).

Figure 1.2. Molecular structure of reduced and oxidised glutathione.

A

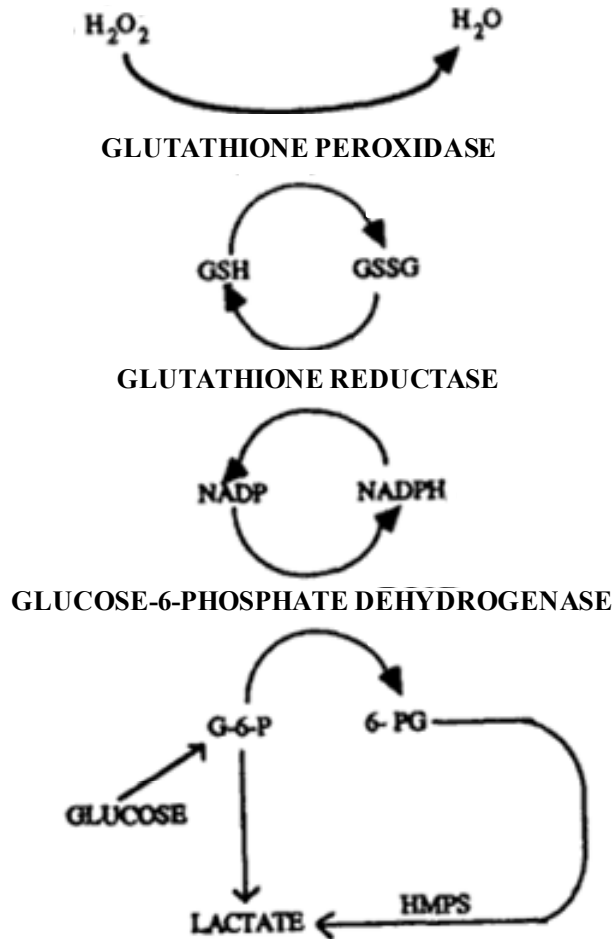


B



Molecular structure of [A] reduced, and [B] oxidised glutathione.

Figure 1.3. Metabolic pathways involved in the reduction of free radicals by glutathione.



Free radicals such as H_2O_2 are inactivated by reduced glutathione through glutathione peroxidase. Oxidised glutathione is reduced by glutathione reductase using NADPH as substrate. NADPH is in turn regenerated by glucose-6-phosphate dehydrogenase, generating 6-phosphogluconate which is metabolised to lactate through the hexose monophosphate shunt or pentose phosphate pathway.

GSH, reduced glutathione; GSSG, oxidised glutathione; G-6-P, glucose-6-phosphate; 6-PG, 6-phosphogluconate; HMPS, hexose monophosphate shunt or pentose phosphate pathway.

CHAPTER 2

MATERIAL AND METHODS

CHAPTER 2

MATERIAL AND METHODS

2.1 ANIMAL MODELS

All experiments were approved under the United Kingdom Home Office regulations for Animals (Scientific Procedures) Act 1986.

2.1.1 Intestinal ischaemia and reperfusion in adult rats

Adult male Sprague-Dawley rats (250 to 300g) were used. The rats were kept under standardised conditions for food, water, light, and temperature. General anaesthesia was induced and maintained throughout the experiments. In the experiments described in Chapter 4, anaesthesia was induced by 2% halothane and oxygen and nitrous oxide (50:50) by inhalation for 5min, and confirmed by absence of webfoot reflex. Anaesthesia was then maintained by 0.5-1.0% halothane inhalation with oxygen and nitrous oxide (50:50) via a nose cone (Harvard Apparatus, Holliston, MA, USA). In the experiments described in Chapter 6, 3% isoflurane and oxygen and nitrous oxide (50:50) by inhalation for 5min was used for induction, followed by 2% isoflurane inhalation with oxygen and nitrous oxide (50:50) via a nose cone.

Immediately after induction, a rectal temperature probe connected to a servo system (CWE Inc, Ardmore, PA, USA) was placed, and body temperature was measured throughout the experiment and maintained within the desired range by use of a lamp and a heating blanket controlled by the servo system.

Once adequacy of general anaesthesia was confirmed by absence of webfoot reflex, the peritoneal cavity was entered via a midline laparotomy. The superior mesenteric artery was then identified (Figure 2.1A) and dissected (Figure 2.1B), and a silicone loop (Surg-I-Loop; Scanlan, MN) was slung around its origin (Figure 2.1C and 2.1D). In animals undergoing sham operation, the loop was immediately removed without occluding the artery, and arterial pulsation on the mesentery was confirmed under direct vision. In rats subjected to intestinal I/R, occlusion of the superior mesenteric artery was performed by continuous traction

(ischaemia) and removal (reperfusion) of the silicone loop slung around the origin of the artery for the assigned duration. The traction was always performed in the same direction and with similar tension in every animal, and efficacy of arterial occlusion was documented by absence of arterial pulsation in the vascular arcades of the mesentery. The peritoneal cavity and the abdominal wall were then closed with a single layer running suture. At the end of the period allocated for mesenteric ischaemia, the suture was removed and the origin of the superior mesenteric artery was once again exposed. In rats undergoing sham operation, pulsation of mesenteric arteries was once again confirmed, and the peritoneum and abdominal wall were closed with a single layer running suture without performing any further intervention. In animals subjected to intestinal I/R, absence of mesenteric flow was confirmed before removing the silicone loop from the origin of the superior mesenteric artery, and reperfusion was then assessed by visualization of pulsation in the vascular arcades of the mesentery. The peritoneum and abdominal wall were closed as previously described. At the end of the experiment, the thorax was entered via a midline sternotomy, the ascending aorta and the superior vena cava were identified and divided, and animals were sacrificed by exsanguination under terminal anaesthesia. The appearance of the bowel at different stages of the procedure is shown in Figure 2.2.

2.1.2 Intestinal ischaemia and reperfusion in infant rats

Suckling Sprague-Dawley rats (11-13d, 20-30g, mixed sex) were housed with their mother and allowed to suckle freely until the beginning of the experiment. General anaesthesia was induced by 3% isoflurane and oxygen and nitrous oxide (50:50) by inhalation for 5min, and confirmed by absence of webfoot reflex. Anaesthesia was then maintained by 1-1.5% isoflurane inhalation with oxygen and nitrous oxide (50:50) via a nose cone (Harvard Apparatus, Holliston, MA, USA). Immediately after induction, a rectal temperature probe connected to a servo system (CWE Inc, Ardmore, PA, USA) was placed, and body temperature was measured throughout the experiment and maintained within the desired range by use of a lamp and a heating blanket controlled by the servo system.

Operative procedures were carried out under microscopic view using an

operating microscope (SZ4045, Olympus, Hamburg, Germany) at 10x magnification. The peritoneal cavity was accessed through a supraumbilical transverse laparotomy (Figure 2.3 A). The superior mesenteric artery was then identified (Figure 2.3 B) and dissected (Figure 2.3 C). In rats subjected to sham operation, no vascular occlusion was performed and the peritoneum and the abdominal wall were closed in one layer with a running suture. In animals undergoing intestinal I/R, occlusion of the superior mesenteric artery was performed by placing two microvascular clips (S&T AG, Switzerland) at its origin (Figure 2.3 D). Complete occlusion of the superior mesenteric artery was confirmed under microscopic view by absence of arterial pulsation in the vascular arcades of the mesentery. The peritoneal cavity and the abdominal wall were then closed with a single layer continuous suture. At the end of ischaemia time, the suture was removed and the origin of the superior mesenteric artery was once again exposed. In rats undergoing sham operation, pulsation of mesenteric arteries was once again assessed under microscopic view, and the peritoneum and abdominal wall were closed as previously described. In animals receiving intestinal I/R, absence of mesenteric flow was confirmed under microscopic view and the microvascular clips at the origin of the superior mesenteric artery were removed. Return of vigorous pulsation in the vascular arcades of the mesentery confirmed reperfusion of the superior mesenteric artery. The peritoneum and abdominal wall were then closed in one layer. At the end of the experiment, the thorax was opened through a midline sternotomy, the ascending aorta and the superior vena cava were identified and divided, and animals were sacrificed by exsanguination. The appearance of the bowel from infant rats at different stages of the procedure is shown in Figure 2.4.

2.2 QUANTIFICATION OF MYELOPEROXIDASE

Neutrophil sequestration in lungs and ileum was estimated by measurement of myeloperoxidase (MPO) activity as described by Bradley et al (Bradley et al, 1982). Tissue samples (~0.2g of left lung and ileum 10-15cm from the ileocaecal valve) were homogenised using an Ultra-Turrax homogeniser in 10mL 50mmol/l

potassium phosphate buffer, pH 6.0, containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide, 100 μ L removed for protein estimation and the remainder centrifuged at 40,790g for 30min. A total of 100 μ L of supernatant then was added to 2.9ml buffer containing 0.53mmol/l *O*-dianisidine hydrochloride and 0.0005% hydrogen peroxide and MPO activity followed spectrophotometrically at 25°C at a wavelength of 460nm. Myeloperoxidase activity was expressed as units (U) per mg protein.

To exclude any possible influence of circulating neutrophils into MPO activity measured in tissue, regression analysis of MPO activity vs. haemoglobin content in tissues was performed, and no correlation was found.

2.3 QUANTIFICATION OF PROTEIN

Protein concentration in tissue homogenate was measured by the method of Peterson (Peterson, 1977). 10 μ L of tissue homogenate were mixed with 990 μ L distilled water, 250 μ L CTC reagent (12.5mmol/L CuSO₄, 20mmol/L sodium tartrate, 2.12mol/L Na₂CO₃), 250 μ L 5% sodium dodecyl sulphate, 250 μ L 0.8mol/L NaOH, and 250 μ L distilled water, and incubated at room temperature for 10 minutes. 500 μ L 16.7% Folin-Ciocalteu reagent in distilled water were added, and incubated at room temperature for further 30 minutes. Absorbance was measured spectrophotometrically at 725nm and compared to that of standard solutions of diluted bovine serum albumin.

2.4 QUANTIFICATION OF MALONDIALDEHYDE

Levels of malondialdehyde (MDA) in plasma and tissues (liver, lungs and kidneys) were measured by high-performance liquid chromatography (HPLC). Tissue samples (~0.2g of liver, left lung, left kidney and ileum 10-15cm from the ileocaecal valve) were homogenised using an Ultra-Turrax homogeniser in 2ml 50mM potassium phosphate buffer (pH 6.0) containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide. Protein concentration of the homogenate was measured by the method of Peterson (Peterson, 1977). 25 μ L of plasma/tissue

homogenate were incubated with 2µl 0.2% (wt/vol) butylated hydroxytoluene in ethanol and 375µl 1% (vol/vol) phosphoric acid, and then derivatised with 345µl 15mmol/l 2-thiobarbituric acid at 100°C for 60min. 200µl of the derivatised solution were collected and mixed with 200µl methanol. After addition of 15µl 1mol/l KH₂PO₄ and 4µl 2mol/l KOH/2.4mol/l KHCO₃ samples were centrifuged (13,000rpm for 10min at 4°C). HPLC was performed on a Hypersil 5µODS column at a flow rate of 1ml/min, isocratically with an eluant of 65% 50mmol/l KH₂PO₄ (pH 7.0)/35% methanol. Fluorescence was monitored by a Jasco FP-1520 detector (excitation wavelength 515nm; emission wavelength 553nm) and values of molar concentration were calculated by comparison with reference solutions of MDA-tetrabutylammonium salt derivatised and analysed in parallel. MDA was expressed as µmol/mg protein for tissues and as µmol/l for plasma.

Under assay conditions, peaks for MDA were well resolved (Figure 2.5 A), and the standard curve was linear for values of MDA up to 20 µmol/l (Figure 2.5 B). Validity of the standard curve was assessed at every assay by linear regression; values of $r^2 > 0.9$ were considered satisfactory. Intra-assay variability was assessed for plasma (n=12, coefficient of variation=4.97%), and intestine (n=12, coefficient of variation=4.89%), lung (n=12, coefficient of variation=6.47%), heart (n=12, coefficient of variation=5.84%), kidney (n=12, coefficient of variation=5.51%) and liver (n=12, coefficient of variation=3.35%) homogenate.

2.5 QUANTIFICATION OF GLUTATHIONE

The concentration of reduced (GSH) and oxidised (GSSG) glutathione in liver, lungs, kidneys and intestine was measured by HPLC as previously described by Reed et al (Reed et al, 1980). Tissue samples (0.7-1.0g of liver, right lung, right kidney and ileum 15 to 25cm from the ileocaecal valve) were weighed and extracted in 2ml 10% perchloric acid/20mmol/l 1,10-phenanthroline, and 100µl 10mmol/l γ-glutamyl-glutamate internal standard was added. The samples were homogenised and centrifuged (3,000rpm for 10min at 4°C). 250µl of supernatant were incubated with 25µl 96.2mmol/l iodoacetic acid (pH 8.5) for 20 min in the dark and derivatised overnight in the dark after addition of 500µl 1% (v/v) 1-

fluoro-2,4-dinitrobenzene. After centrifugation of the samples (13,000rpm for 10min at 4°C) HPLC was performed at a flow rate of 1.2 ml/min on a Hypersil 5 μ APS-2 column. Initial conditions were: 5% buffer (36% 2M sodium acetate in methanol) against 76% methanol followed by a gradient to 31.4% buffer against 55% methanol over 33min, then to 44% buffer against 45% methanol over 2min and to 100% buffer over 15min, which was continued for 5min. Absorbance was measured at a wavelength of 359nm by a Jasco MD-910 detector. Molar concentration of GSH and GSSG in the samples was calculated according to the recovery rate of the internal standard. Concentrations of GSH, GSSG and total glutathione (calculated as GSH + 2GSSG) in tissue were expressed as nmol/g wet weight. Glutathione redox state was calculated as ratio of GSH/GSSG.

Under assay conditions peaks for γ -glutamyl-glutamate, GSH and GSSG were well resolved in intestine, lungs and liver extracts (Figure 2.6). However, analysis of kidney extracts did not yield clearly detectable peaks of GSH and GSSG, and data on kidney glutathione content are therefore not available.

2.6 QUANTIFICATION OF NITRATE PLUS NITRITE

The plasma concentration of nitrate plus nitrite (nitrate + nitrite), reflecting systemic production of nitric oxide (NO), was measured by HPLC using a novel highly sensitive method.

The stock solution for nitrite was prepared by dissolving 690mg of sodium nitrite in 100ml Milli-Q water to obtain a 100mmol/l primary standard. Similarly, a 100mmol/l stock solution for nitrate was obtained by dissolving 849.9mg of sodium nitrate in 100ml Milli-Q water. Nitrite and nitrate standards (200, 150, 125, 100, 75, 50, 25 and 10 μ mol/l) were prepared by subsequent dilutions of the relative stock solutions. 5 μ l of standard or plasma was placed in a 400- μ l autosampler vial. 20 μ l 68.75 μ mol/l NADPH and 20 μ l 825U/l nitrate reductase were added to each vial. The vials were gently mixed and incubated at room temperature for 180 min to allow a complete reduction of nitrate to nitrite (Giovannoni et al, 1997). 63 μ l of 480 μ mol/l N-methyl-4-amino-7-nitrobenzofurazan (MNBDH) in acetonitrile and 67 μ l of 20% v/v H₃PO₄ were added to each vial. The vials were mixed again and

derivatised at room temperature for 30 min. 20 μ l was injected directly into the HPLC.

HPLC of MNBDH was modified from Buldt and Karst (Buldt et al, 1999). The flow rate was 0.62ml/min, and a gradient of 50mmol/l ammonium acetate (pH 7.5) against acetonitrile was run on a Hypersil 5 μ ODS column with a Phenomenex SecurityGuard ODS cartridge. Initial conditions were: 45% buffer and 55% acetonitrile, for 1.5min, followed by a gradient to 100% acetonitrile 8.5min, which was continued for 4min. The column effluent was monitored by a fluorescence detector (Jasco FP-1520) at an excitation wavelength of 468nm and an emission wavelength of 537nm. Plasma concentrations of nitrate + nitrite were expressed as μ mol/l.

Addition of ammonium acetate to 50mM improved peak shape and lengthened column life. MNBDH was well resolved from the solvent front and from interfering peaks (Figure 2.7 A). The standard curve of nitrite was linear up to at least 200 μ mol/l (Figure 2.7 B). Enzymatic conversion of nitrate to nitrite using nitrate reductase was undertaken, based on the conversion conditions used by Giovannoni et al (Giovannoni et al, 1997), and the standard curve of nitrate after conversion with nitrate reductase was linear and identical to that of nitrite run in parallel, reflecting complete conversion of nitrate to nitrite. There was a significant peak for nitrite even in the zero standard, equivalent to \sim 100 μ mol/l nitrite, which presumably originates from nitrite in the reagents, or reaction of MNBDH with atmospheric NO. This peak did not originate from nitrite in the Milli-Q water used, as blanks run in the absence of the 5 μ l zero standard had an identical sized peak. In addition, there was a consistent difference between the zero nitrate standard and the zero nitrite standard, equivalent to \sim 4 μ mol/l nitrite, presumably due to presence of nitrite or nitrate in the NADPH or nitrate reductase used for the enzymatic conversion. Due to these confounding factors, it is important to run suitable standards in parallel with each set of samples analysed.

Using this method, control adult rat plasma had a nitrate + nitrite concentration of $45.5 \pm 2.4 \mu\text{mol/l}$ (n=10) and 11-13d old rats $101.5 \pm 7.0 \mu\text{mol/l}$ (n=3). Adult human plasma from healthy volunteers had a nitrate + nitrite concentration of $67.4 \pm 10.1 \mu\text{mol/l}$ (n=6), whereas children studied before elective

operation for gastro-oesophageal reflux had a nitrate + nitrite concentration of $44.1 \pm 7.3 \mu\text{mol/l}$ ($n=6$).

Intra-assay variability, as assessed by measuring the average coefficient of variation of adult human plasma samples run in duplicate in the same assay ($n=5$), was 5.44%. Inter-assay variability, as assessed by measuring the average coefficient of variation of adult human plasma samples run on two different days ($n=5$), was 3.67%.

2.7 QUANTIFICATION OF TNF- α

Concentration of TNF- α in plasma was measured with a rat TNF- α ELISA kit (BioSource International, Camarillo, CA, USA), and optical density was read at 450nm on a microtitre plate reader against a series of reference solutions (minimum detectable dose=4pg/ml). TNF- α concentration in plasma were expressed as pg/ml.

2.8 QUANTIFICATION OF PHOSPHOENERGETICS

Concentrations of adenosine tri-phosphate (ATP), adenosine di-phosphate (ADP), and adenosine mono-phosphate (AMP) were measured in liver extracts by HPLC (Figure 328).

A liver sample (~0.03g) was ground under liquid nitrogen and extracted with 600 μl 12% perchloric acid in a mortar. The homogenate was centrifuged at 13,000rpm for 10min and the supernatant was collected and neutralised to pH 4.0-6.0 with 2mol/l KOH/2.4mol/l KHCO_3 . The samples were centrifuged at 13,000rpm for 10min; 100 μl of supernatant was analysed by HPLC at 0.7 ml/min on a HyPurity Aquastar 5 μODS column, isocratically with an eluant of 120 $\mu\text{mol/l}$ KH_2PO_4 pH6.0. Absorbance was measured at a wavelength of 260nm by a Jasco MD-910 detector. Values of molar concentration of ATP, ADP, and AMP in the samples were calculated by comparison with reference solutions (Sigma-Aldrich Co. Poole, Dorset, UK) analysed in parallel. Concentrations of ATP, ADP and AMP in liver were expressed as $\mu\text{mol/g}$ wet weight. Total adenine nucleotides

concentration was calculated as ATP+ADP+AMP, and liver energy charge as $(\text{ATP} + \frac{1}{2}\text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})$.

Concentrations of inorganic phosphate were measured in the supernatant using a commercially available kit (Sigma-Aldrich Co. Poole, Dorset, UK) and expressed as $\mu\text{mol/g}$ wet weight.

2.9 QUANTIFICATION OF CARNITINE PALMITOYL TRANSFERASE ACTIVITY

Activity of carnitine palmitoyl transferase I (CPT I) or total carnitine palmitoyl transferase (CPT) activity were measured in cardiac tissue obtained at the end of experiments.

2.9.1 Assay for carnitine palmitoyl transferase I activity

At the end of the experiment, the chest was opened and heart was immediately removed and placed in ice-cold medium containing 120mmol/l KCl, 20mmol/l 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5mmol/l MgCl_2 , 1mmol/l ethylene glycol tetraacetic acid (EGTA), 5mg/mL fatty acid free bovine serum albumin (BSA) pH 7.4 followed by homogenisation in a polytron homogenizer and isolation of mitochondria by differential centrifugation as previously described (Eaton et al, 1993). Heart mitochondria were greater than 90% intact as judged by citrate synthase latency (Shepherd et al, 1969).

CPT I activity was measured radiochemically as previously described (Grantham et al, 1986; Fukumoto et al, 2002a) in a medium consisting of 80mmol/l KCl, 10mmol/l HEPES, 5mmol/l MgCl_2 , 2.5mmol/l KH_2PO_4 , 1mmol/l EGTA, 30mmol/l phosphocreatine, 20mmol/l creatine, 31U/mL creatine phosphokinase, 1mmol/l ATP, 0.1mmol/l coenzyme A (CoA), 0.62 $\mu\text{mol/l}$ myxothiazol, 135 $\mu\text{mol/l}$ palmitoyl-CoA, and 1mmol/l ^3H -carnitine pH 7.2. CPT I activity was calculated in terms of nmol/min/U citrate synthase.

Citrate synthase activity was measured by adding: 10 μl mitochondria suspension diluted 1:10 in a medium consisting of 250mmol/L sucrose, 2mmol/L HEPES, and 0.1mmol/L EGTA pH 7.4; 10 μl 5mmol/L Acetyl-CoA in 50mmol/L KH_2PO_4 pH 5.3; 10 μl 10mmol/L DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) in

Tris (2-amino-2-hydroxymethyl-propane-1,3-diol) HCl; 5µl 0.05mol/L oxaloacetic acid in Tris-HCl; and 955µl Tris-HCl. Absorbance was measured at 412nm by spectrophotometry before and after adding 10µl Triton 10% to evaluate integrity of mitochondria (at least 90% intact mitochondria was considered adequate).

2.9.2 Assay for total carnitine palmitoyl transferase activity

At the end of the experiment, the chest was opened and the heart was immediately removed and freeze-clamped in liquid nitrogen to arrest the metabolism, and then stored at -80°C until analysis. Heart samples were thawed and homogenised with a polytron homogeniser in a medium consisting of 80mmol/l KCl, 10mmol/l HEPES, 5mmol/l MgCl₂, 2.5mmol/l KH₂PO₄, 1mmol/l EGTA, 30mmol/l phosphocreatine, 20mmol/l creatine, 31U/mL creatine phosphokinase, 1mmol/l ATP, 0.1mmol/l CoA, 0.62µmol/l myxothiazol (pH 7.2). Three cycles of freezing in liquid nitrogen and thawing were performed to ensure blowing of mitochondrial membranes. 135µmol/l palmitoyl-CoA, and 1mmol/l ³H-carnitine were then added to the homogenate, and total CPT activity was measured as previously described and calculated in terms of nmol/min/U citrate synthase.

Citrate synthase activity was measured by adding: 10µl 5mmol/L Acetyl-CoA in 50mmol/L KH₂PO₄ pH 5.3; 10µl 10mmol/L DTNB in Tris-HCl; 10µl Triton 10%; 100µl heart homogenate diluted 1/100 in a medium consisting of 250mmol/L sucrose, 2mmol/L HEPES, and 0.1mmol/L EGTA pH 7.4; 5µl 0.05mol/L oxaloacetic acid in Tris-HCl; and 865µl Tris-HCl. Absorbance was measured at 412nm by spectrophotometry.

2.10 HISTOLOGICAL INJURY IN THE ILEUM

Terminal ileum (5 to 10cm from the ileocaecal valve) was fixed in 10% formaldehyde for 24 hours and embedded in paraffin. 5µm sections were cut and stained with haematoxylin and eosin.

Histological injury was evaluated and graded blindly by three investigators (including the author) using a well established scoring system (Figure 2.9) (Vejchapipat et al, 2002): 1) Normal mucosal villi; 2) Development of mucosal

slough at villous tips; 3) Extension of the sub-epithelial space with the epithelial layer lifting up in sheets, presence of a few denuded villous tips, and mild capillary congestion; 4) Denuded villi with exposed lamina propria, dilated, exposed capillaries with evidence of haemorrhage, and increased cellularity of the lamina propria; 5) Digestion and disintegration of the lamina propria and presence of haemorrhage and ulceration.

2.11 ADHESION MOLECULE EXPRESSION IN THE ILEUM

Immunostaining for P-Selectin (CD62P), ICAM-1 (CD54, cluster of differentiation 54) and V-CAM-1 (CD106, cluster of differentiation 106) were performed on 5µm sections of paraffin-embedded terminal ileum obtained as described previously.

2.11.1 *P-selectin (CD62P)*

Antigen retrieval was achieved by microwave heating in 1% citrate buffer pH 6.0. After blockade of endogenous peroxidase activity with 3% hydrogen peroxide in phosphate buffered saline (PBS) and incubation with 10% normal goat serum, sections were incubated with primary antibody [P-Selectin (CD62P), BD Biosciences, San Jose, CA, USA, 1:200 dilution in PBS] overnight at 4°C, after which anti-rabbit labelled polymer horseradish peroxidase (HRP) and diaminobenzidine (DAB) (EnVision, DakoCytomation, Cambridge, UK) were applied.

2.11.2 *ICAM-1 (CD54)*

After microwave heating in 1% citrate buffer pH 6.0, blockade of endogenous peroxidase activity and incubation with 10% normal goat serum were performed. Primary antibody [ICAM-1 (CD54), Endogen, Woburn, MA, USA, 1:2,000 dilution in PBS] was applied for 60min at room temperature, and sections were incubated with anti-mouse labelled polymer HRP and DAB (EnVision, DakoCytomation, Cambridge, UK).

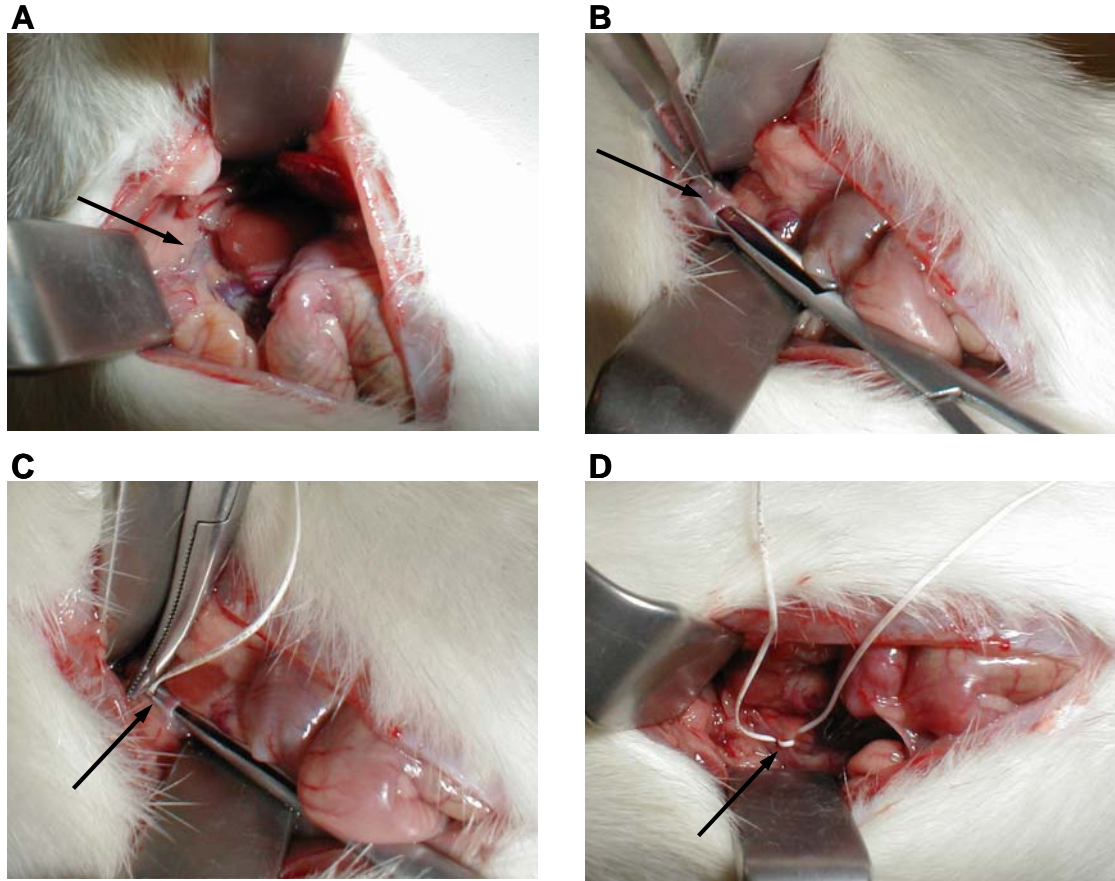
2.11.3 VCAM-1 (CD106)

Sections underwent blockade of endogenous peroxidase activity and incubation with 10% normal goat serum and were incubated with VCAM-1 primary antibody [VCAM-1 (CD106), BD PharMingen, San Diego, Ca, USA, 1:15,000 dilution in PBS] for 60min at room temperature. Anti-mouse labelled polymer HRP and DAB were then applied.

2.10.4 Quantification of adhesion molecule expression

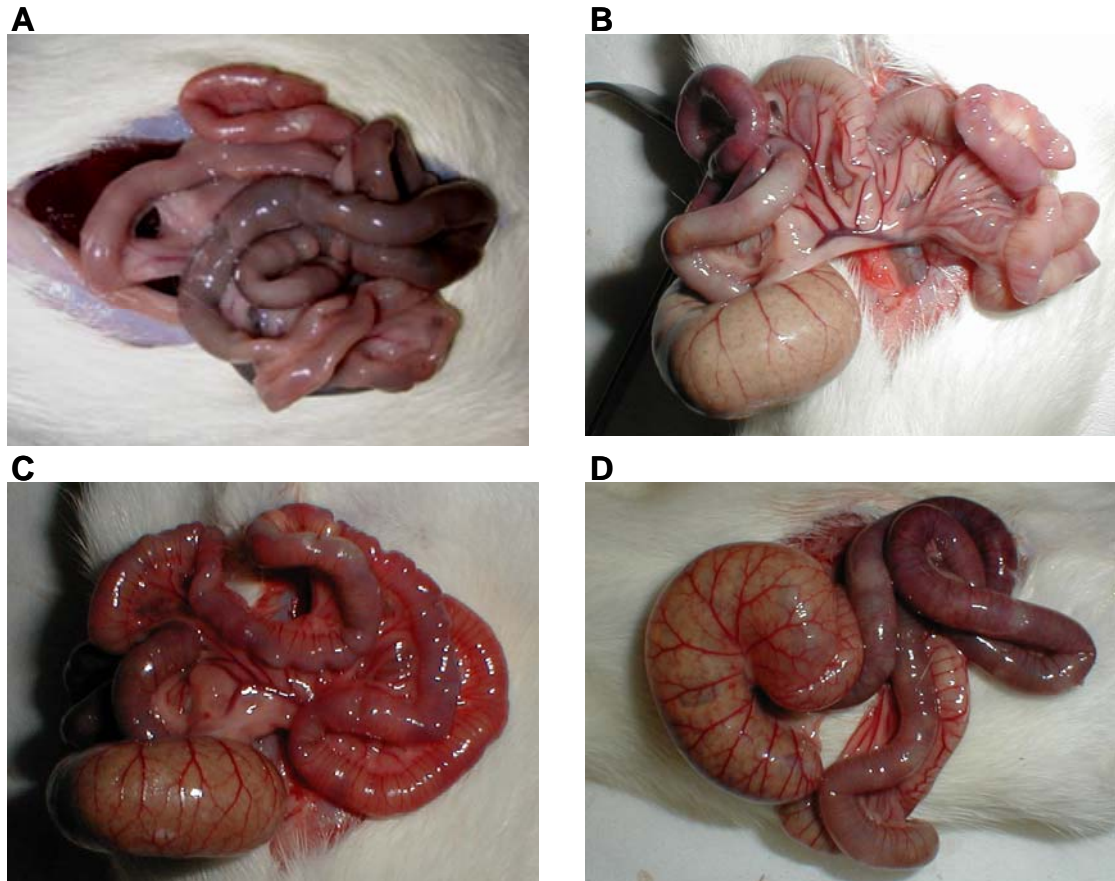
Expression of P-selectin, ICAM-1 and VCAM-1 was assessed by grading the staining intensity of the endothelium of subepithelial vessels. The specimens were scored blindly by three investigators (including the author) on a semi-quantitative scale based both on the number of positive vessels and on the intensity of the staining: 1) Weak staining; 2) Mild staining; 3) Moderate staining; 4) Strong staining.

Figure 2.1. Surgical procedure in adult rats.



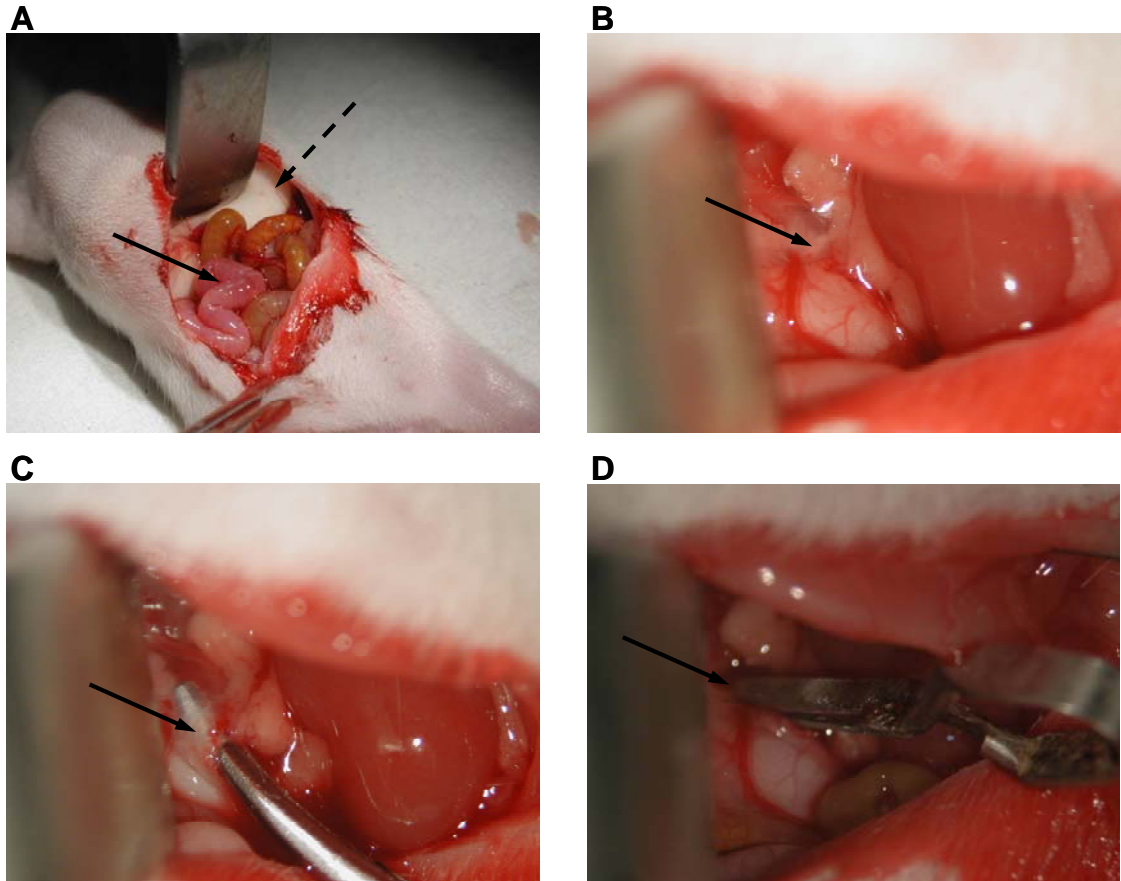
After accessing the peritoneum through a midline incision, the superior mesenteric artery (arrow) is identified [A] and dissected at its origin from the abdominal aorta [B]. A silicon loop (Surg-I-Loop; Scanlan, MN) is then passed behind the origin of the superior mesenteric artery [C] and slung around the artery [D].

Figure 2.2. Appearance of the bowel from adult rats at different stages.



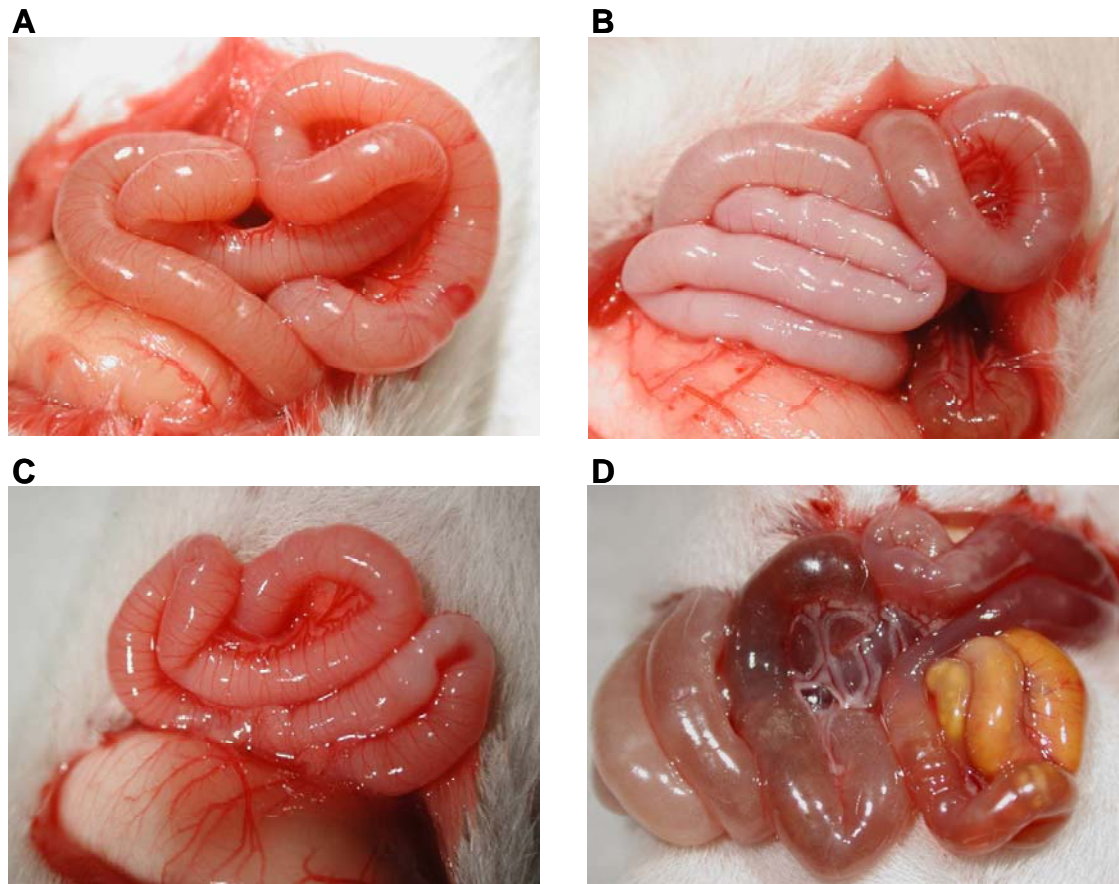
[A] Normal blood perfusion is present at the beginning of the experiment, before intestinal ischaemia is performed. [B] At the end of the ischaemic phase, prolonged absence of blood flow results in diffuse pallor of the ileum, with initial signs of necrosis (top left corner). [C] Diffuse hyperaemia and congestion of blood vessels is visible at the very beginning of reperfusion, which is confirmed by vigorous pulsation of the arterial arcades of the mesentery. [D] At the end of the reperfusion phase, most of the terminal ileum appears frankly necrotic with evidence of intraluminal haemorrhage (top right corner), whereas the colon (to the left) appears relatively preserved.

Figure 2.3. Surgical procedure in infant rats.



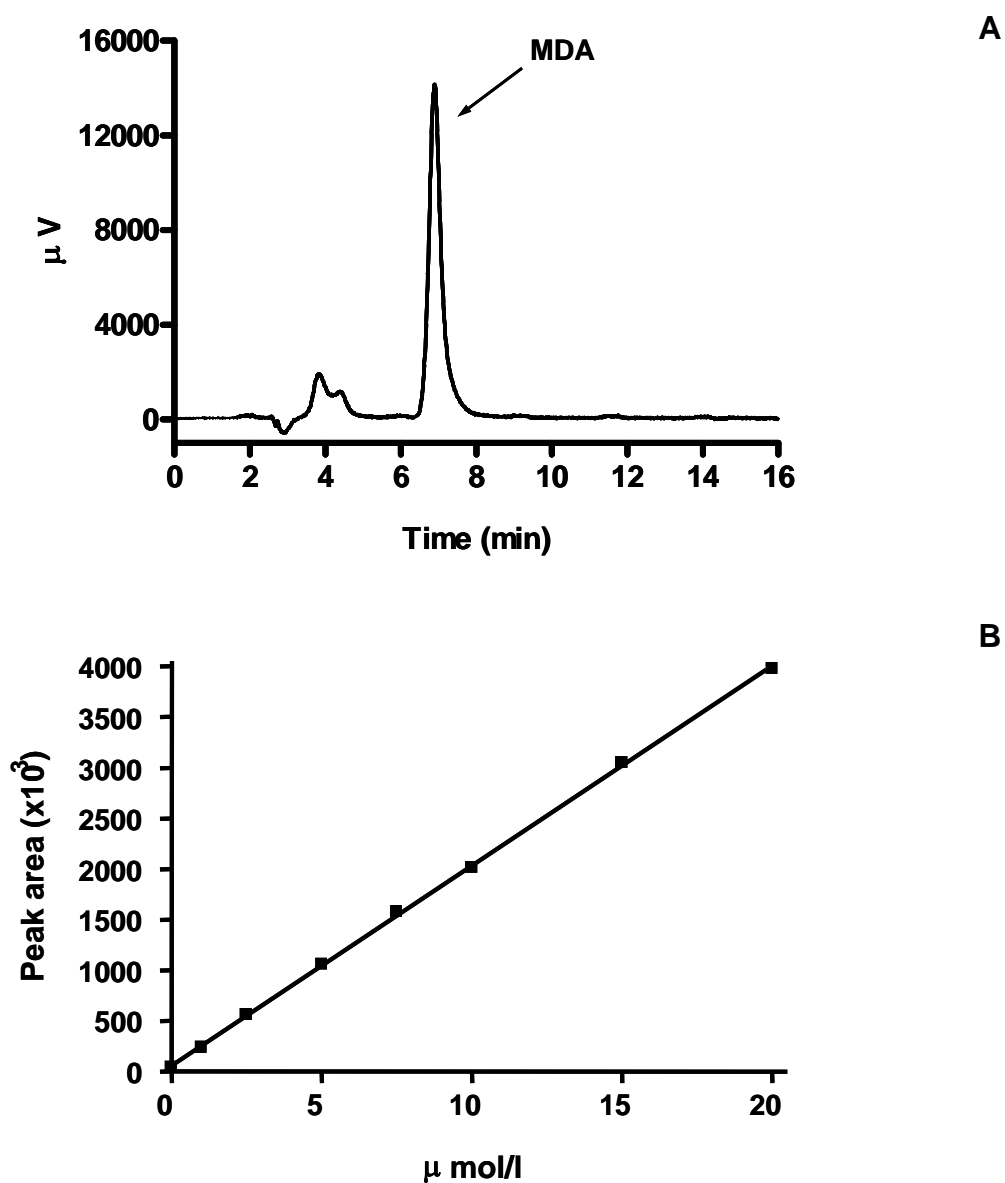
After accessing the peritoneal cavity through a supraumbilical transverse laparotomy, the stomach (dashed arrow) and the small intestine (arrow) are retracted [A]. Under microscopic view (magnification 10x), the superior mesenteric artery (arrow) is identified [B] and dissected at its origin from the abdominal aorta [C], and the first of two microvascular clips (S&T AG, Switzerland) is then placed around the origin of the superior mesenteric artery (arrow) [D].

Figure 2.4. Appearance of the bowel from infant rats at different stages.



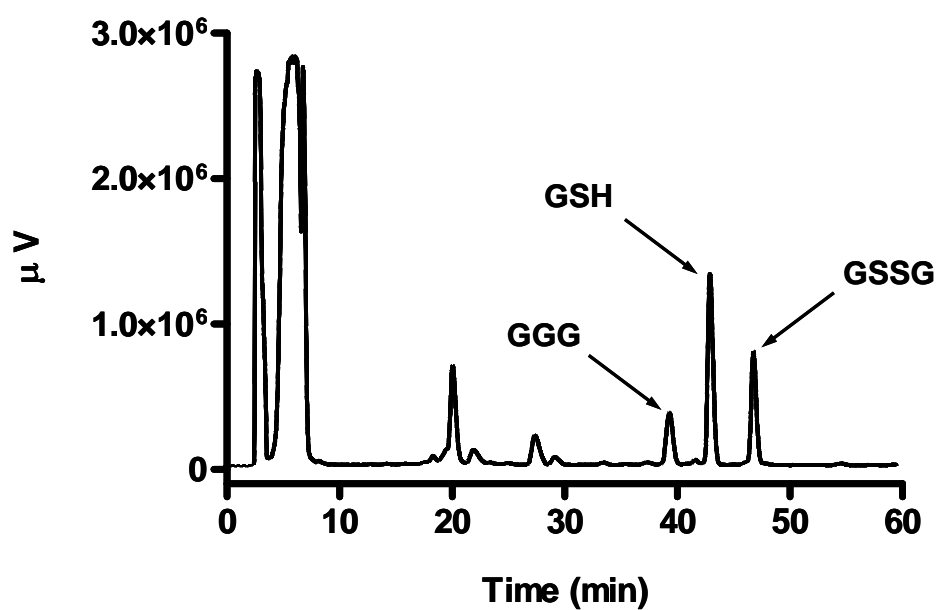
[A] Normal blood perfusion is present at the beginning of the experiment, before intestinal ischaemia is performed. [B] At the end of the ischaemic phase, prolonged absence of blood flow results in diffuse pallor of the ileum, with initial signs of necrosis (top right corner). [C] Diffuse hyperaemia and congestion of blood vessels is visible at the very beginning of reperfusion, which is confirmed by vigorous pulsation of the arterial arcades of the mesentery under microscopic view. [D] At the end of the reperfusion phase, part of the terminal ileum appears frankly necrotic with evidence of intraluminal haemorrhage.

Figure 2.5. Malondialdehyde assay for rat plasma sample.



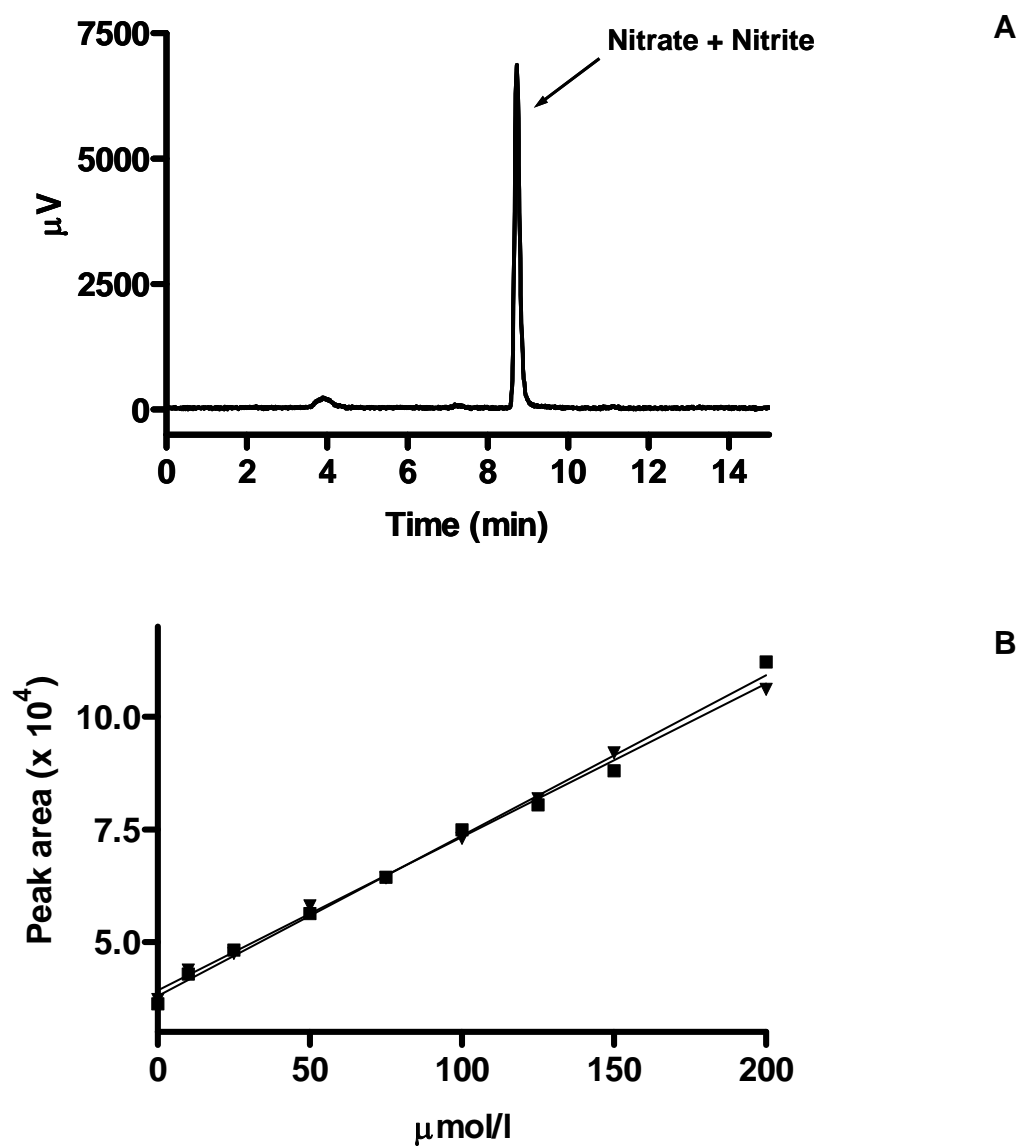
[A] Chromatogram of malondialdehyde (MDA) assay for rat plasma sample. Sample was derivatised and run as described. The peak for MDA is indicated by the arrow. [B] Standard curve for MDA. Samples were derivatised and run as described. Curve fitting was by linear regression ($r^2=0.9997$).

Figure 2.6. Glutathione assay for rat intestine sample.



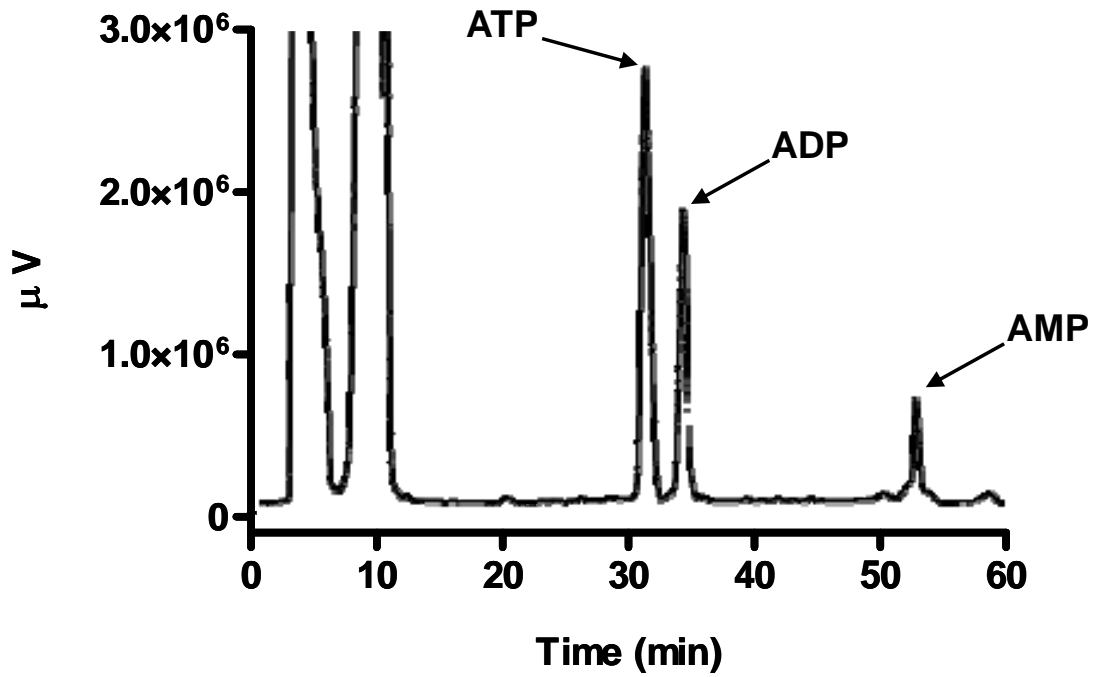
Chromatogram for glutathione assay for rat intestine sample. The sample was derivatised and run as described. The peaks for γ -glutamyl-glutamate (GGG), reduced glutathione (GSH), and oxidised glutathione (GSSG) are indicated by arrows.

Figure 2.7. Nitrate plus nitrite assay for rat plasma sample.



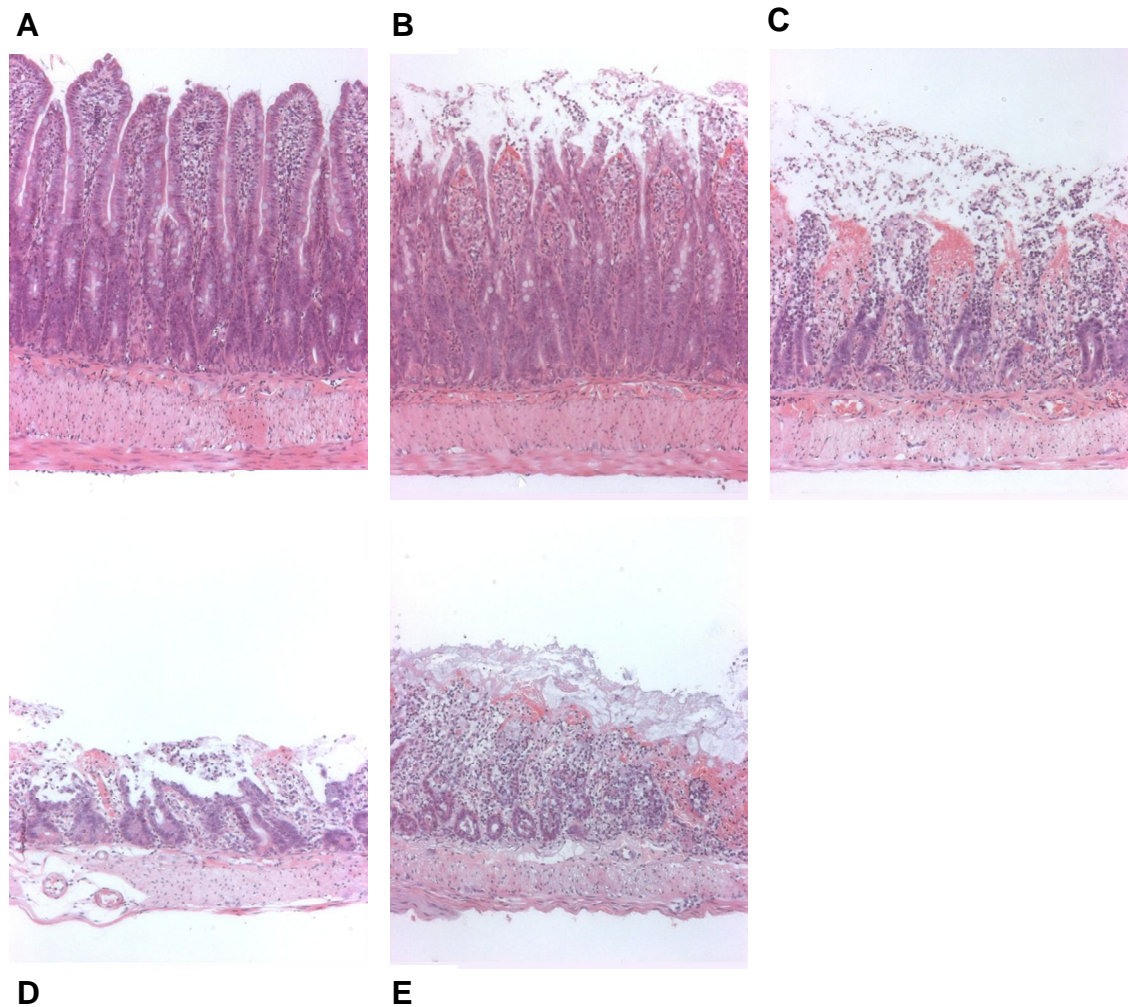
[A] Chromatogram for nitrate plus nitrite for rat plasma sample. The sample was derivatised and run as described. [B] Standard curves of [■] nitrite and [▼] nitrate. Samples were derivatised and run as described in the materials and methods. Curve fitting was by linear regression (nitrate: $r^2=0.9935$; nitrite: $r^2=0.9914$).

Figure 2.8. Phosphoenergetics assay for rat liver sample.



Chromatogram for phosphoenergetics assay for rat liver sample. The sample was derivatised and run as described. The peaks for adenosine tri-phosphate (ATP), adenosine di-phosphate (ADP), and adenosine mono-phosphate (AMP) are indicated by arrows.

Figure 2.9. Grading of histological injury in the ileum.



Grading of histological injury on haematoxylin and eosin stained terminal ileum (samples are from adult rats) (Vejchapipat et al, 2002). Grade 1: normal mucosa [A]; Grade 2: mucosal slough at villous tips [B]; Grade 3: denuded villous tips, and mild capillary congestion [C]; Grade 4: denuded villi, and dilated capillaries [D]; Grade 5: haemorrhage and ulceration [E].

CHAPTER 3

MODERATE HYPOTHERMIA AS A PREVENTATIVE TREATMENT FOR ADULT INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

CHAPTER 3

MODERATE HYPOTHERMIA AS A PREVENTATIVE TREATMENT FOR ADULT INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

3.1 BACKGROUND

Intestinal I/R injury represents a major clinical problem in infants, children and adults. Diseases associated with this condition include necrotizing enterocolitis, midgut volvulus, acute mesenteric arterial occlusion, haemodynamic shock and sepsis (Schoenberg et al, 1993), as discussed in Chapter 1. Intestinal I/R can also occur as a consequence of surgical procedures such as cardiopulmonary bypass, aortic aneurysm repair and intestinal transplantation (Soong et al, 1994; Gennaro et al, 1993; Sola et al, 2001).

A life threatening consequence of intestinal I/R is the development of MODS (Neary et al, 1999) characterised by impairment of respiratory (Harward et al, 1993; Iglesias et al, 1998), cardiocirculatory (Horton et al, 1991), hepatic (Vejchapipat et al, 2001; Turnage et al, 1996) and renal (LaNoue et al, 1996) function. The pathogenesis of multiple organ failure after intestinal I/R is multifactorial, and the development of a systemic inflammatory response with subsequent oxidative injury appears to play a pivotal role in this setting (Cuzzocrea et al, 2000; Kalia et al, 2001). When the amount of reactive oxygen and nitrogen species produced by different sources overwhelms the scavenging potential of endogenous antioxidant systems (Li et al, 2002), leading to a state of oxidative stress, cell injury and death develop as a consequence of protein modification, lipid membrane disruption and DNA damage (Tan et al, 1999; Cuzzocrea et al, 2001b).

Previous studies have shown that hypothermia during experimental intestinal I/R injury prevents liver bioenergetic failure and mortality (Vejchapipat et al, 2001), and that hypothermia is beneficial not only in reducing local damage (Hassoun et al, 2002; Vejchapipat et al, 2002; Kalia et al, 2002b), but also in decreasing lung neutrophil infiltration (Vinardi et al, 2003), and the effect on mortality has been confirmed (Kalia et al, 2001).

The purpose of this study was to investigate the consequences of intestinal I/R and the effects of whole-body moderate hypothermia on systemic oxidative stress in an adult rat model, by measuring the extent of lipid peroxidation and the content of glutathione, and by assessing systemic production of NO.

3.2 MATERIAL AND METHODS

3.2.1 Study design

Young adult male Sprague-Dawley rats weighing between 250 and 300g were used. Animals were kept under standard conditions for food, water, light and temperature, and had access to standard rat chow and water *ad libitum* until the beginning of the experiment.

Animals were randomly assigned to one of four experimental groups (n=10 per group):

- A) Control Normothermia: sham operation for 180min at normothermia
- B) I/R Normothermia: 60min intestinal ischaemia, followed by 120min reperfusion at normothermia
- C) Control Hypothermia: sham operation for 180min at moderate hypothermia
- D) I/R Hypothermia: 60min intestinal ischaemia, followed by 120min reperfusion at moderate hypothermia

Rectal temperature was monitored continuously throughout the experiment from induction of anaesthesia. Normothermia (rectal temperature between 36 and 38°C) was maintained by use of a heating blanket and lamp as described in Chapter 2. Moderate hypothermia (rectal temperature between 30 and 32°C) was induced immediately after induction of anaesthesia by exposing the rats to an environmental temperature of 22 to 23°C, with warming by a heating blanket and lamp to prevent excessive cooling. All animals were allowed to stabilise for a period of 30min after anaesthesia and monitoring had been established, in order to achieve the desired rectal temperature.

3.2.2 Surgical Procedure

General anaesthesia was performed by inhalation of halothane in an oxygen and nitrous oxide mixture as described in Chapter 2.

Once adequacy of general anaesthesia was confirmed, superior mesenteric artery ischaemia and reperfusion, and sham operation were performed as described in Chapter 2.

At the end of the experiment, the thorax was entered via a midline sternotomy, the ascending aorta and the superior vena cava were identified and divided, and animals were sacrificed by exsanguination under terminal anaesthesia.

3.2.3 Tissue extraction and metabolite measurement

Immediately after sacrifice, the heart was removed and placed in ice-cold medium for CPT I activity measurement. A blood sample was collected from the thorax into a heparin tube and centrifuged at 3,000rpm for 5 min at 4°C; plasma was separated and stored at -70°C. Lungs, liver, kidneys and two samples of terminal ileum (10 to 15cm and 15 to 25cm from the ileocaecal valve) were removed and stored at -70°C until analysis.

Levels of MDA in plasma, terminal ileum, liver, lungs and kidneys were measured. The concentration of GSH and GSSG in liver, lungs, kidneys and intestine was measured by HPLC. Plasma concentration of nitrate plus nitrite, reflecting systemic production of NO, was measured by HPLC. CPT I activity in the heart was measured by radiochemical assay; heart samples from 8 rats per group only were analysed. Assays for MDA, glutathione, nitrate plus nitrite, and CPT I were performed as described in Chapter 2.

3.2.4 Statistical analysis

Data are expressed as mean \pm SEM (standard error of the mean). One-way analysis of variance (ANOVA) with Tukey post hoc test was used for group comparison. Linear regression was used to analyse the relationship between levels of MDA and nitrate plus nitrite concentration in plasma. Results showing p values <0.05 were considered significant.

3.3 RESULTS

3.3.1 *Lipid peroxidation*

Intestinal I/R at normothermia caused a marked increase in malondialdehyde in plasma (Figure 431), ileum (Figure 3.2 A) and lungs (Figure 3.2 B) compared to controls. Moderate hypothermia prevented the effects of reperfusion injury: the degree of lipid peroxidation in plasma, ileum and lungs after hypothermic I/R was significantly lower than after normothermic I/R and comparable to both normothermic and hypothermic controls (Figure 3.1, Figure 3.2 A, Figure 3.2 B).

In kidneys, a slight but statistically not significant increase in MDA levels was observed following intestinal reperfusion (Figure 3.2 C). However, hypothermic I/R animals had significantly decreased kidney MDA compared to I/R normothermia.

Consequences of intestinal reperfusion in the liver differed from plasma, ileum, lungs and kidneys: lipid peroxidation was not increased in animals undergoing intestinal I/R compared to controls (Figure 3.2 D).

3.3.2 *Glutathione content*

Intestinal levels of reduced, oxidised and total glutathione (Figure 3.3 A, B, C) were profoundly decreased in normothermic animals as a consequence of intestinal ischaemia and reperfusion injury, reflecting a marked impairment in cellular antioxidant status. Hypothermic I/R rats showed a milder reduction in gut GSH compared to normothermic I/R animals, and levels of GSSG comparable to those of normothermic controls (Figure 3.3 A, B). Unexpectedly, moderate hypothermia induced an increase in ileal GSH and total glutathione content in the absence of intestinal ischaemia (Figure 3.3 A, C). Glutathione redox state was grossly affected in the ileum after I/R at normothermia with respect to controls, whereas it was unaffected in hypothermic I/R rats (Figure 3.3 D). Ischaemia and reperfusion injury did not affect glutathione levels in either liver or lungs.

Although no decrease in liver glutathione levels or redox state was observed following intestinal reperfusion (Figure 3.4), moderate hypothermia had a similar

effect on liver glutathione levels to that observed in ileum: a significant increase in total glutathione (Figure 3.4 C) was observed, which was mostly due to an increase in GSSG (Figure 3.4 B).

Glutathione content in lungs was unaffected by either intestinal reperfusion, or moderate hypothermia (Figure 3.5).

3.3.3 Systemic nitric oxide production

There was a significant increase in systemic nitric oxide production (as indicated by plasma nitrate + nitrite concentration) following normothermic intestinal I/R with respect to controls (Figure 3.6). This effect was not observed at moderate hypothermia: NO synthesis in rats undergoing hypothermic I/R was similar to that of controls at either hypothermia or normothermia (Figure 3.6). Interestingly, a highly significant positive correlation was observed between systemic NO production and plasma levels of MDA between all samples (Figure 3.7).

3.3.4 Carnitine palmitoyl transferase I activity in the heart

The activity of cardiac CPT I was significantly impaired after I/R at normothermia compared to normothermic control animals (Figure 3.8). However, moderate hypothermia appeared to prevent this effect: CPT I activity in hypothermic rats subjected to I/R was considerably higher than in normothermic I/R animals and similar to controls at both normothermia and moderate hypothermia. In control rats, no significant differences were observed between animals maintained at normothermia and moderate hypothermia.

4.4 DISCUSSION

Restoration of blood circulation to ischaemic tissue is essential to re-establish oxygen and nutrient supply and therefore to prevent the progression of cell injury. However, reperfusion is associated with a series of physiological processes that can ultimately lead to paradoxical tissue damage, as previously discussed in Chapter 1. As soon as reoxygenation is restored after a period of anoxia, a wide

range of ROS (such as hydrogen peroxide, superoxide, hydroxyl and perhydroxyl radicals) and RNS (nitric oxide, peroxynitrite) species are produced by activated neutrophils (Cuzzocrea et al, 2001b), xanthine oxidase (Terada et al, 1992), mitochondria and other intracellular sources (Li et al, 2002). Reactive species are highly unstable molecules that can oxidise membrane phospholipids through the self-propagating chain reaction process of lipid peroxidation (Tribble et al, 1987) and damage other biomolecules, including proteins and nucleic acids (Cuzzocrea et al, 2001b). Biological protection against these reactive species consists of an integrated system of enzymatic and chemical antioxidants for detoxification of oxidative and nitrosative compounds, but an imbalance between oxidant production and antioxidant defences can result in oxidative stress and subsequent tissue damage (Comhair et al, 2002). Following visceral I/R injury, oxidative stress develops not only at a local level (Hassoun et al, 2002; Sola et al, 2000) but also in distant organs as a consequence of systemic inflammatory response driven by activation of neutrophils and production of inflammatory mediators (Foulds et al, 1998; Barry et al, 1997; Carden et al, 1993).

The increase in lipid peroxidation end-products in plasma observed in this experiment confirms this hypothesis, but the degree of lipid peroxidation is different in the organs I studied. Intestine and lungs exhibited a profound degree of lipid peroxidation following reperfusion, whereas in kidneys only a mild enhancement in lipid peroxidation was noticed and no difference was seen in liver. These divergent effects may reflect a different degree of inflammatory infiltrate in distant organs, as suggested by an increase in TNF- α in the lungs, but not in liver and kidneys, after intestinal I/R (Rahat et al, 2001). Alternatively, the antioxidant defences of liver and kidney may be sufficient to prevent lipid peroxidation of biological membranes. This hypothesis is supported by the finding that although intestinal glutathione status is profoundly decreased by I/R, that of liver is unaltered. This is also supported by the observation that in a model of intestinal I/R in which hepatic glutathione status was altered, hepatic lipid peroxidation also occurred (Turnage et al, 1991). Glutathione is active in the reduced form, and becomes oxidised as it protects against reactive species. Oxidised glutathione can be recycled back to reduced, active, glutathione, so that in tissues undergoing

oxidative stress, the total glutathione pool may be unaltered, but the reduced vs. oxidised redox state is altered. Intestinal I/R induced not only a decrease in the amount of reduced glutathione and in glutathione redox state, but also a diminution of the total pool of glutathione. This phenomenon has been previously observed in intestinal reperfusion injury (Gibson et al, 1993; Bhaskar et al, 1995) and reflects an irreversible loss of glutathione, possibly by reaction of reduced glutathione with hypochlorous acid (Pullar et al, 2001) or degradation to cystine (Bhaskar et al, 1995).

Nitric oxide is an endogenously produced reactive molecule with important biological functions such as regulation of vascular contractility. In addition to these regulatory functions, it may have other deleterious effects, for example overproduction can lead to cellular damage, either directly, through combination with superoxide to produce the potentially cytotoxic peroxynitrite, or by the end products of its production, nitrate and nitrite. These facets of NO are also observed in intestinal reperfusion injury: NO may have local beneficial effects within the intestine during I/R, via mechanism including vasodilatation and inhibition of leukocyte adherence to the endothelium (Chan et al, 1999; Horie et al, 1998; Kalia et al, 2002a) whereas systemic effects of NO and/ or its end products are deleterious and can lead to multiple organ dysfunction (Yang et al, 2002). I did not measure local NO production or nitric oxide synthase activity in this model, but instead measured plasma nitrate plus nitrite as a measure of systemic NO production. The systemic deleterious effects of nitric oxide and/or its products are supported in this study by the strong positive correlation between plasma nitrite + nitrate with plasma MDA (Figure 3.7).

Hypothermia exerted a crucial effect in maintaining intestinal antioxidant potential: preservation of the endogenous pool of reduced glutathione is critical to antioxidant defences (Rahman et al, 2000) and a decrease in glutathione redox state has been suggested to be connected to the development of apoptotic and necrotic cell death (Schafer et al, 2001). In fact, glutathione redox state is considered to be the primary determinant of cell redox state, because it is 3 to 4 orders of magnitude more abundant than the other redox couples (such as NADH/NAD^+ , NADPH/NADP^+ , cysteine/cystine, thioredoxin_{red}/thioredoxin_{oxid}, gltaredoxin_{red},

glutaredoxin_{oxid}), and because it has a low standard redox potential. In turn, cell reduction potential plays a crucial role in regulating cellular status, including transition to proliferation, differentiation, apoptosis, and necrosis - the latter being associated with high values of cell reduction potential (and therefore low values of redox state) (Schafer et al, 2001). A reduction in the amount of oxidants, due to a decrease in leukocyte activation (Vinardi et al, 2003) and cytokine pro-inflammatory response (Williams et al, 2002) may in part explain the protective effect of hypothermia observed in my study, but the increase in intestinal and hepatic glutathione concentrations observed in hypothermic controls compared to normothermic controls suggests the existence of an alternative mechanism. The finding that hypothermia alone increased gut glutathione levels is novel. Previous studies have suggested that intestinal glutathione may be depleted during chronic cold stress (Kaushik et al, 2003; Simmons et al, 1990; Spasic et al, 1993). However, the intestinal activity of γ -glutamyl-cysteine synthetase, the rate-controlling enzyme of glutathione synthesis, appears to be able to be upregulated acutely in response to water immersion stress (Alptekin et al, 1996) so that increased glutathione synthesis could be responsible for the increased glutathione concentration observed. The improved glutathione status of hypothermic animals undergoing intestinal I/R injury may play an important role in prevention of propagation of a systemic inflammatory response, as well as decreasing localised tissue damage.

Average reduced glutathione concentrations in my experiment was 380 nmol/g wet weight in the small bowel, 890 nmol/g wet weight in the liver, and 275 nmol/g wet weight in the lung, and average glutathione redox state was 3.6, 2.1, and 0.9 respectively. Absolute values of reduced glutathione concentration in rat tissues vary significantly in the literature, from as low as 1.3 (Jewell et al, 1999) to as high as 1580 nmol/g wet weight (Kuhn et al, 2000). However, glutathione redox state is more consistent, and typically between 4:1 and 10:1 in rat small intestine when calculated as $[GSH]/2*[GSSG]$ (Tian J et al, 2007; Tian J et al, 2009; Wu et al, 2004), and comprised between 15:1 and 50:1 at a cellular level (Schafer et al, 2001). Values of glutathione redox state averaged 3.6 in control animals in my study, which is lower than what reported by most authors but comparable to what

reported in some series (Wu et al, 2004). Although partial oxidation of glutathione may have possibly occurred from the time of animal sacrifice to the time of sample analysis in my experiments as a consequence of both autoxidation and enzyme-catalysed degradation, this is unlikely given the stability of reduced glutathione when stored at -80°C (Lin SK et al, 2006).

The beneficial effects of moderate hypothermia on lipid peroxidation in plasma, lungs and kidneys may depend on the potential of hypothermia to reduce leukocyte adherence to the endothelium and neutrophil infiltration in local and distal organs following intestinal I/R (Vinardi et al, 2003; Kalia et al, 2001). In addition, hypothermia has been shown to decrease production of reactive oxygen species by stimulated neutrophils “in vitro” (Sung et al, 1985; Akriotis et al, 1985) and to suppress production of pro-inflammatory cytokines after I/R injury (Qing et al, 2001; Kato et al, 2002). Since whole-body moderate hypothermia was applied in this model, the protective effects observed in distal organs may depend either on the prevention of leukocytes activation and priming in the ileum, or in the reduction of neutrophils recruitment and functions in distant organs.

Previous studies have shown that after intestinal I/R, the isolated perfused heart has impaired contractile function, which can be prevented by free-radical scavengers and inhibitors of neutrophil adherence (Horton et al, 1991; Horton et al, 1993a; Horton et al, 1993b). I/R injury causes release of cytokines and endotoxin into the portal and systemic circulation (Williams et al, 2002) and this is thought to cause recruitment of neutrophils into distal tissues (Schmeling et al, 1989; Vinardi et al, 2003), induction of nitric oxide synthase (Cain et al, 1999), and free radical production. The reaction of nitric oxide and superoxide produces peroxynitrite, which is thought to be important in the pathogenesis of myocardial dysfunction due to inflammatory causes (Ferdinandy et al, 2000; Flesch et al, 1999; Ferdinandy et al, 1999; Khadour et al, 2002; Schulz et al, 1997), decreasing cardiac contractile function by uncoupling ATP production from mechanical work (Schulz et al, 1997).

Fatty acid oxidation is central to cardiac metabolism (Eaton, 2002), providing around 75% of cardiac ATP requirements under most conditions (Neely et al, 1974). CPT I and CPT II control passage of long-chain fatty acids through the

external and internal mitochondrial membrane, and therefore their access to the inner mitochondrial milieu where they become the substrate of the β -oxidation pathway (Figure 3.9). CPT I is important in the control of fatty acid oxidation in the heart, as in other tissues (Eaton, 2002), and it has been previously shown that cardiac CPT I activity is impaired during neonatal sepsis and by peroxynitrite (Fukumoto et al, 2002a) and that this impairment is due to oxidative modification of the enzyme (Fukumoto et al, 2002b). It is possible that intestinal reperfusion injury, by triggering a systemic inflammatory response leading to the development of oxidative stress at a systemic level, would inhibit cardiac CPT I activity similarly to neonatal endotoxaemia. This is supported by the increase in plasma MDA and nitric oxide production secondary to normothermic intestinal reperfusion observed in the present experiment. In this study, cardiac CPT I activity was markedly inhibited by intestinal I/R injury; this would be predicted to decrease myocardial fatty acid oxidation and increase the reliance of the heart on other substrates such as glucose, lactate and ketone bodies. This effect may make the heart more vulnerable to variation in nutritional supply following intestinal I/R.

Intestinal I/R in this model induces intestinal histological changes and energy failure, as well as liver energy failure (Vejchapipat et al, 2002; Vejchapipat et al, 2001; Vejchapipat et al, 2000; Vinardi et al, 2003). In the present experiment, I have additionally shown that CPT I activity was impaired by the effects of intestinal I/R. These suggest that the balance of substrate utilisation was shifted from fatty acid oxidation to either carbohydrate or ketone body utilisation. Using the same animal model, it has been previously shown that moderate hypothermia has beneficial effects on liver function (Vejchapipat et al, 2001) and gut histology (Vejchapipat et al, 2002). Interestingly, moderate hypothermia prevented the inhibition of cardiac CPT I activity after intestinal I/R, together with previous studies showing the benefits of hypothermia to the gut, liver and lungs, supporting the potential clinical utility of moderate hypothermia in amelioration of multi-system organ failure due to intestinal I/R injury.

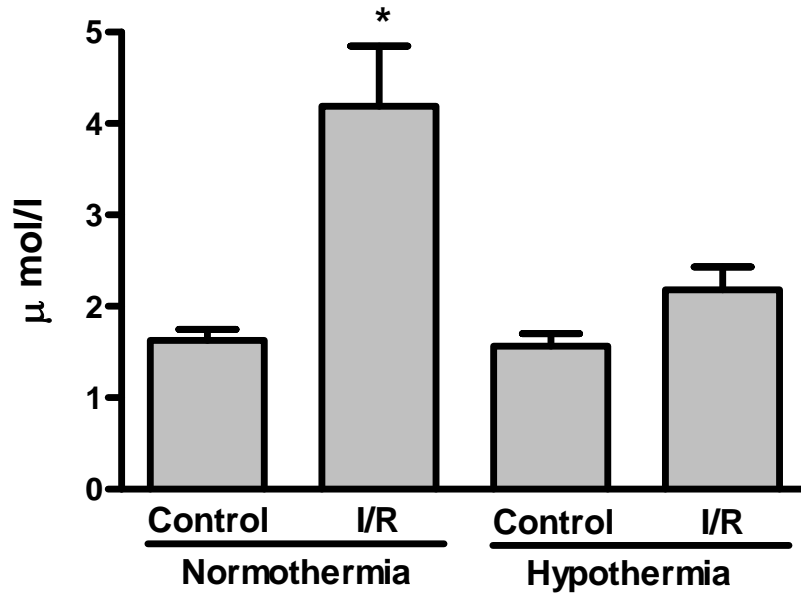
Protection against oxidative damage achieved during ischaemia and early reperfusion may remain effective even after restoration of a normothermic status, although the effects of rewarming on animals undergoing hypothermic I/R were

not evaluated in this study. Restoration of normothermia did not influence mortality and did not jeopardise hypothermia-induced reduction of leukocyte adhesion in intestinal mucosa in a similar animal model (Kalia et al, 2001), suggesting that benefits conferred by hypothermia may persist upon rewarming.

4.5 CONCLUSIONS

This study has shown that intestinal I/R is associated with an increase in lipid peroxidation in plasma, ileum, lungs and, to a lesser extent, kidneys; with a depletion of the intestinal pool of reduced glutathione; with an enhanced systemic nitric oxide production; and with impaired fatty acid metabolism in the heart. Total-body moderate hypothermia applied throughout I/R proved to be effective in counteracting these changes, precluding the development of systemic oxidative stress and therefore preventing the progression of multiple organ failure by blocking one of its major pathways. These results suggest that moderate hypothermia could be beneficial in clinical conditions associated with intestinal I/R injury, especially when intestinal ischaemia can be foreseen and hypothermia can be applied at the beginning of the ischaemic injury.

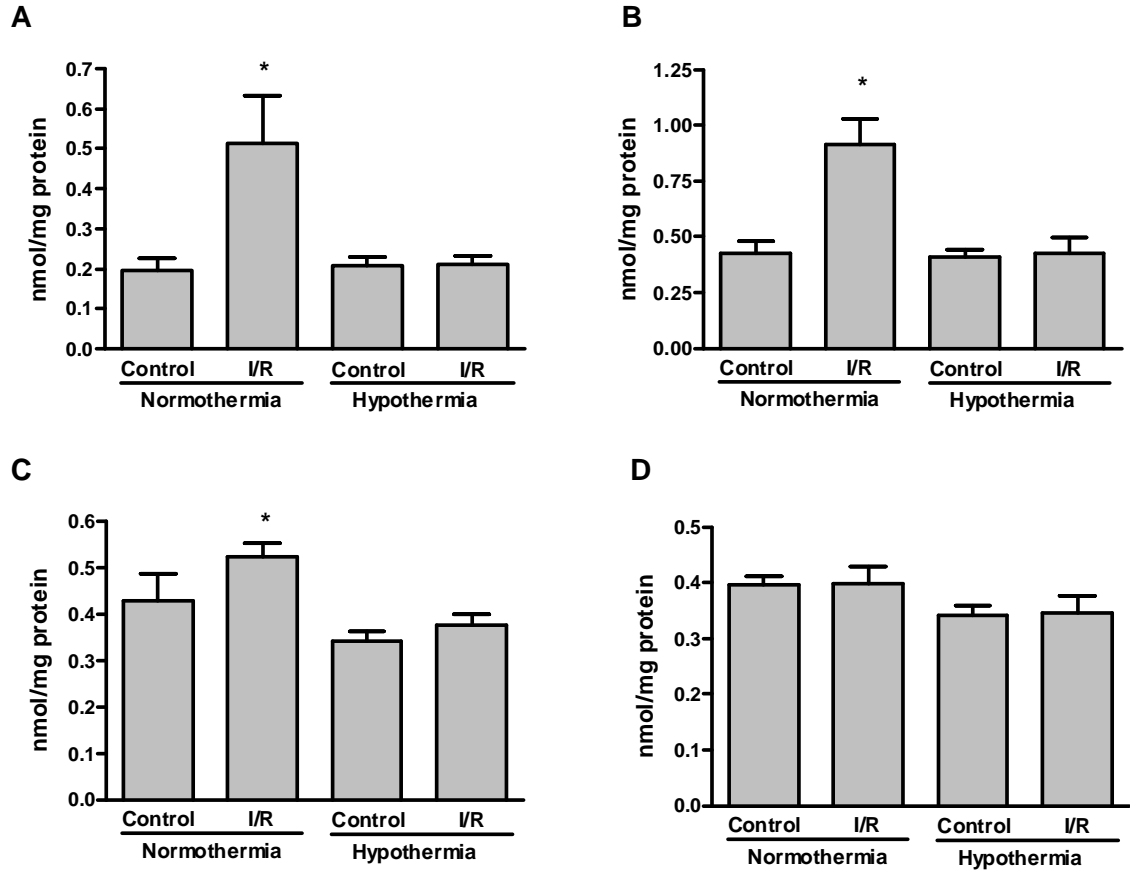
Figure 3.1. Malondialdehyde concentration in plasma.



Concentration of malondialdehyde (MDA) in plasma. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Data are expressed as $\mu\text{mol/l}$ and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

* I/R Normothermia: $P<0.001$ vs. Control Normothermia; $P<0.001$ vs. Control Hypothermia; $P=0.002$ vs. I/R Hypothermia.

Figure 3.2. Malondialdehyde concentration in ileum, lungs, kidneys, and liver.



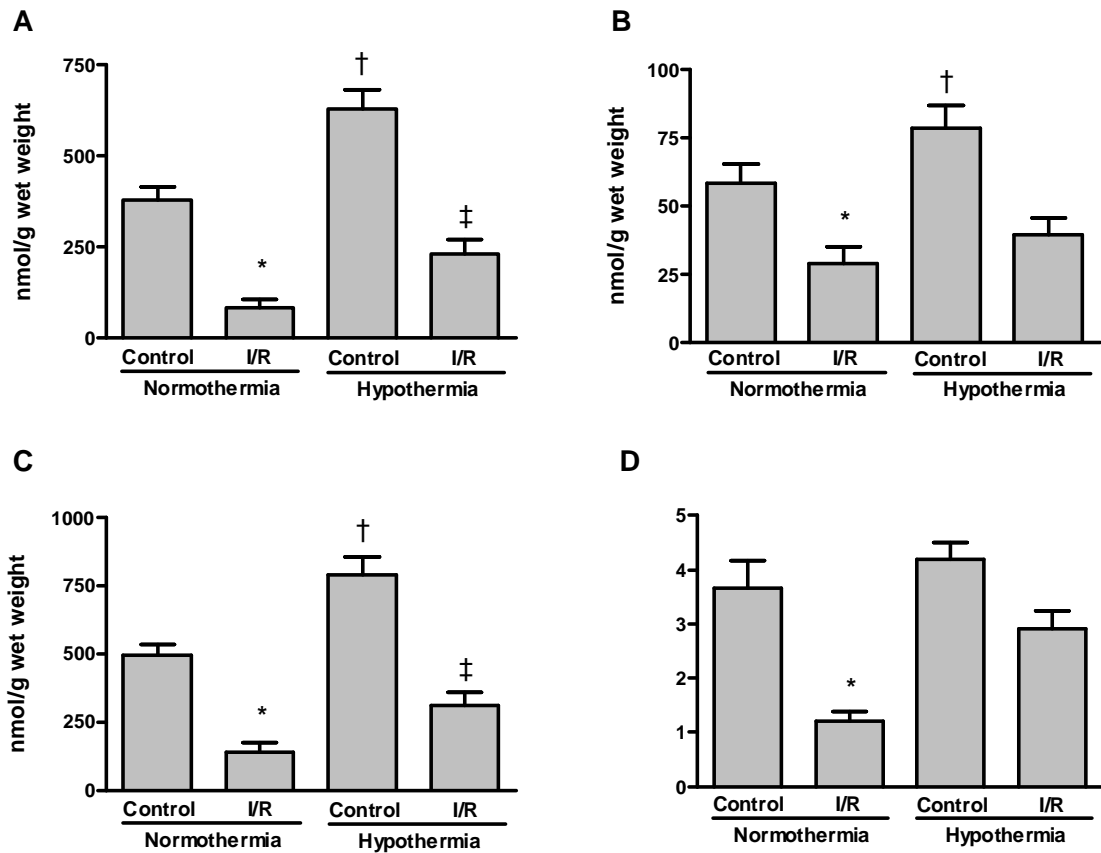
Concentration of malondialdehyde (MDA) in ileum [A], lungs [B], kidneys [C], and liver [D]. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Data are expressed as nmol/mg protein and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Normothermia: $P=0.007$ vs. Control Normothermia; $P=0.010$ vs. Control Hypothermia; $P=0.011$ vs. I/R Hypothermia.

[B]: * I/R Normothermia: $P<0.001$ vs. Control Normothermia; $P<0.001$ vs. Control Hypothermia; $P<0.001$ vs. I/R Hypothermia.

[C]: * I/R Normothermia: $P = 0.006$ vs. Control Hypothermia; $P = 0.035$ vs. I/R Hypothermia.

Figure 3.3. Glutathione concentration in ileum.



Concentration of reduced (GSH) [A], oxidised (GSSG) [B], and total (calculated as $GSH + 2 \times GSSG$) [C] glutathione, and glutathione redox state (calculated as $GSH/2 \times GSSG$) [D] in ileum. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentrations of GSH, GSSG and total glutathione are expressed as nmol/g wet weight. Results are reported as mean \pm SEM; one-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Normothermia: $P < 0.001$ vs. Control Normothermia; $P < 0.001$ vs. Control Hypothermia; $P = 0.048$ vs. I/R Hypothermia.

† Control Hypothermia: $P < 0.001$ vs. Control Normothermia; $P < 0.001$ vs. I/R Hypothermia.

‡ I/R Hypothermia: $P=0.044$ vs. Control Normothermia.

[B]: * I/R Normothermia: $P=0.029$ vs. Control Normothermia; $P<0.001$ vs. Control Hypothermia.

† Control Hypothermia: $P=0.002$ vs. I/R Hypothermia.

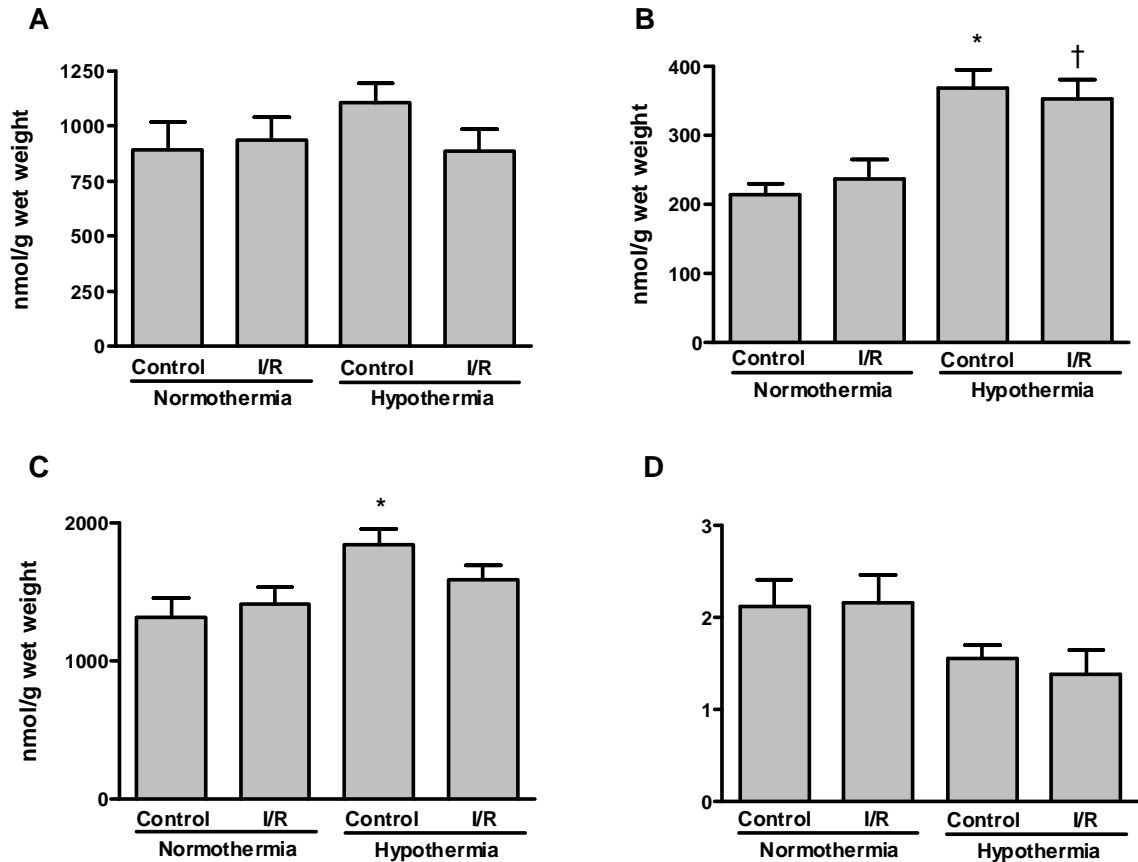
[C]: * I/R Normothermia: $P<0.001$ vs. Control Normothermia; $P<0.001$ vs. Control Hypothermia.

† Control Hypothermia: $P=0.001$ vs. Control Normothermia; $P<0.001$ vs. I/R Hypothermia.

‡ I/R Hypothermia: $P=0.047$ vs. Control Normothermia.

[D]: * I/R Normothermia: $P<0.001$ vs. Control Normothermia; $P<0.001$ vs. Control Hypothermia; $P=0.006$ vs. I/R Hypothermia.

Figure 3.4. Glutathione concentration in liver.



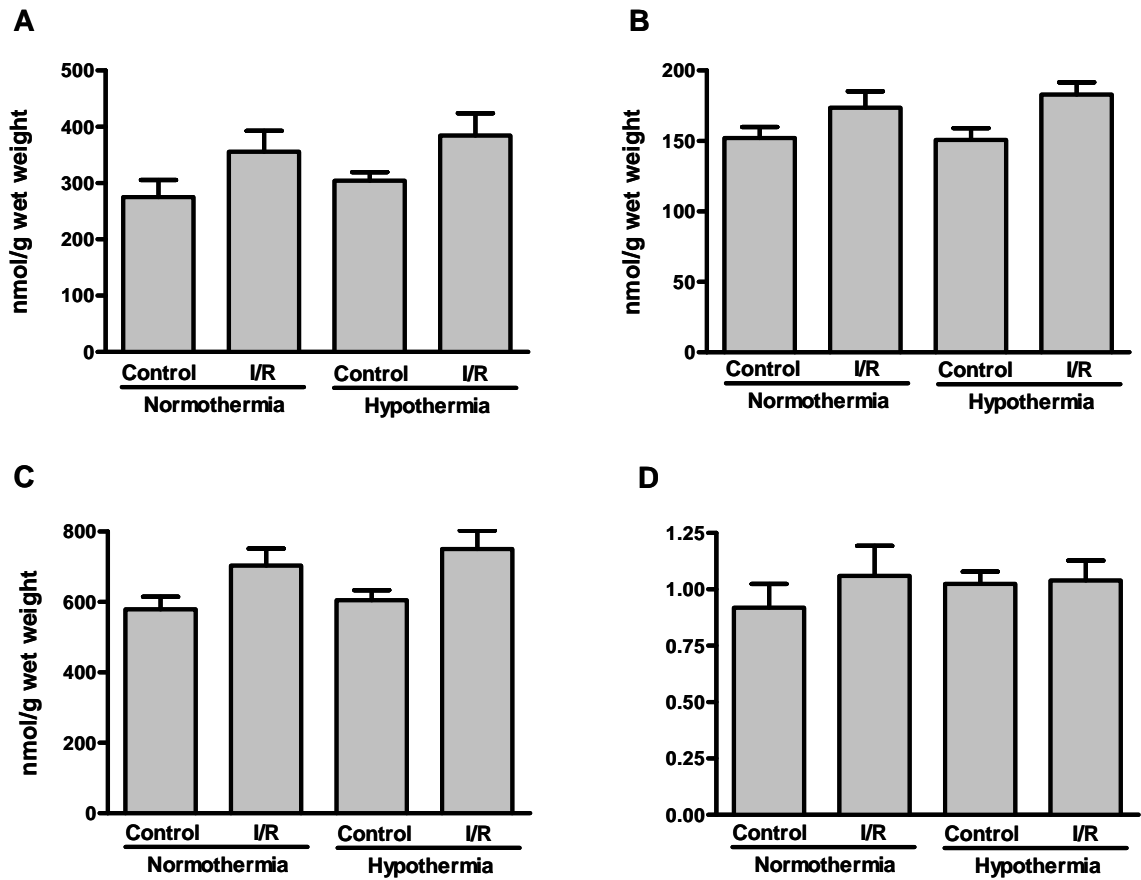
Concentration of reduced (GSH) [A], oxidised (GSSG) [B], and total (calculated as $GSH + 2 \times GSSG$) [C] glutathione, and glutathione redox state (calculated as $GSH/2 \times GSSG$) [D] in liver. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentrations of GSH, GSSG and total glutathione are expressed as nmol/g wet weight. Results are reported as mean \pm SEM; one-way ANOVA with Tukey post hoc test was used for group comparison.

[B]: * Control Hypothermia: $P < 0.001$ vs. Control Normothermia; $P = 0.003$ vs. I/R Normothermia.

† I/R Hypothermia: $P = 0.002$ vs. Control Normothermia; $P = 0.013$ vs. I/R Normothermia.

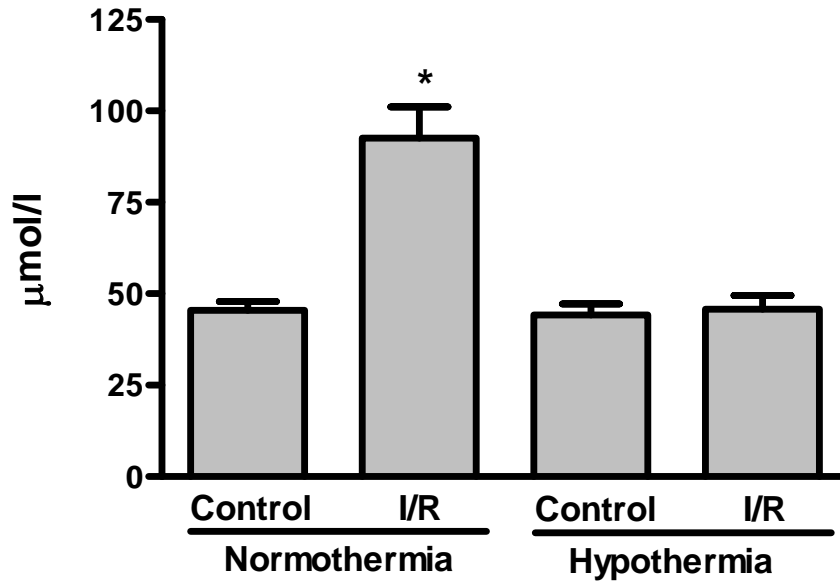
[C]: * Control Hypothermia: $P = 0.21$ vs. Control Normothermia.

Figure 3.5. Glutathione concentration in lungs.



Concentration of reduced (GSH) [A], oxidised (GSSG) [B], and total (calculated as $GSH + 2 \times GSSG$) [C] glutathione, and glutathione redox state (calculated as $GSH/2 \times GSSG$) [D] in lungs. Animals ($n=10$ per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentrations of GSH, GSSG and total glutathione are expressed as nmol/g wet weight. Results are reported as mean \pm SEM; one-way ANOVA with Tukey post hoc test was used for group comparison.

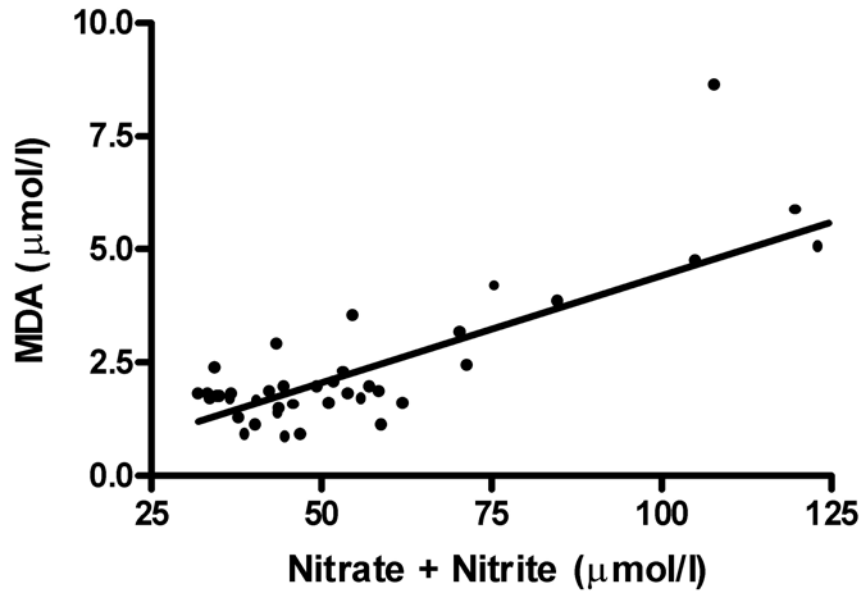
Figure 3.6. Nitrate plus nitrite concentration in plasma.



Systemic production of nitric oxide measured as concentration of nitrate plus nitrite in plasma. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Data are expressed as $\mu\text{mol/l}$ and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

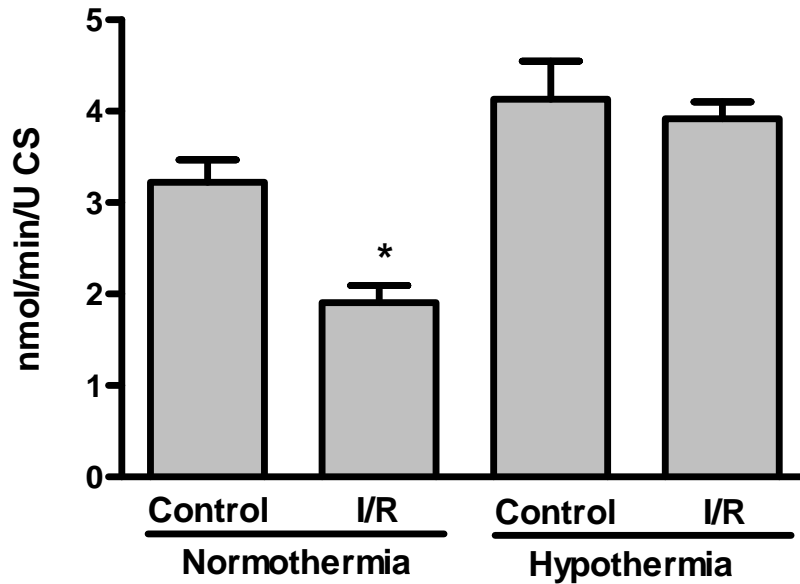
* I/R Normothermia: $P < 0.001$ vs. Control Normothermia; $P < 0.001$ vs. Control Hypothermia; $P < 0.001$ vs. I/R Hypothermia.

Figure 3.7. Correlation between nitrate plus nitrite and malondialdehyde concentration in plasma.



Correlation between nitrate plus nitrite in plasma (expressed as μmol/l) and malondialdehyde (MDA, expressed as μmol/l) concentration in plasma (linear regression analysis indicated a correlation with $r^2=0.630$; $p<0.0001$).

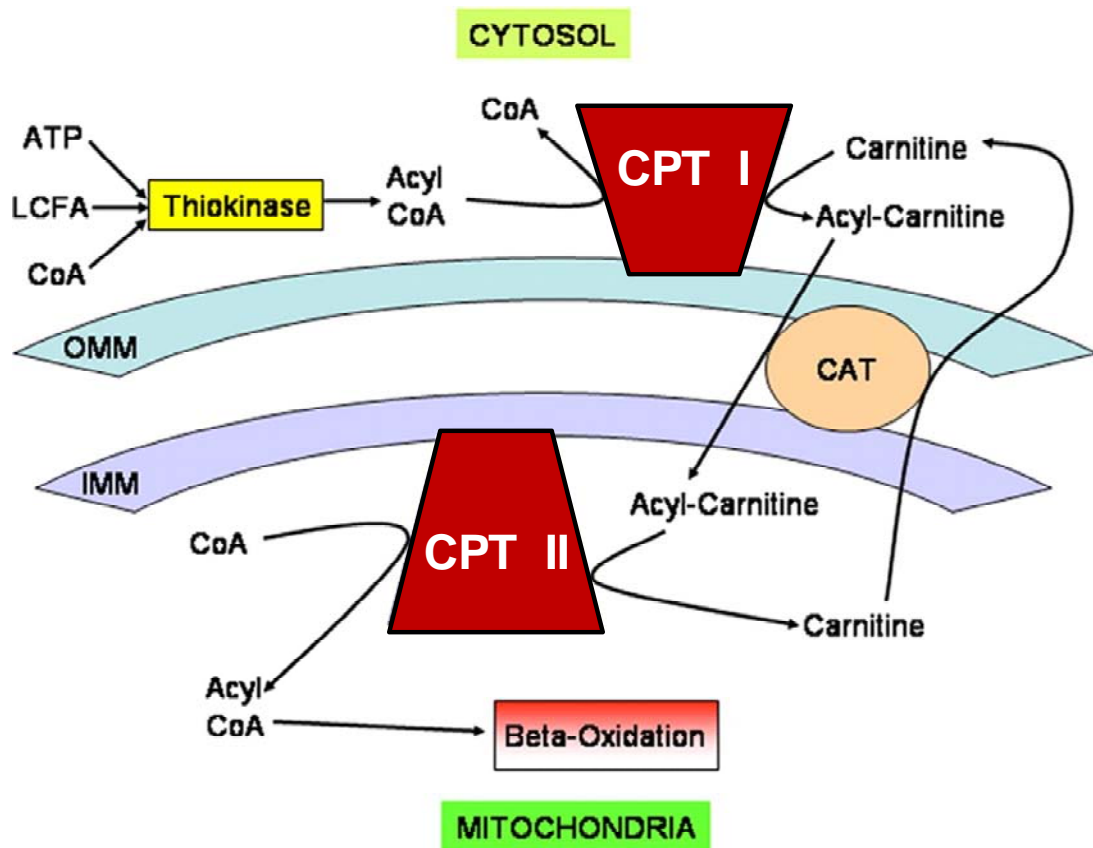
Figure 3.8. Carnitine palmitoyl transferase I activity in the heart.



Carnitine palmitoyl transferase I (CPT I) activity in the heart. Animals (n=8 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Data are expressed as mmol/min/U citrate synthase (CS) and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

* I/R Normothermia: $p=0.012$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia; $p<0.001$ vs. I/R Hypothermia

Figure 3.9. Role of carnitine palmitoyl transferase I and II (CPT I and CPT II) in mitochondrial long-chain fatty acid uptake (Lee et al, 2004).



Schematic representation of mitochondrial long-chain-fatty-acid uptake. CPT I is a point for control and regulation of fatty acid oxidation via modulation of its activity. CAT, carnitine-acylcarnitine-translocase; CPT I, carnitine-palmitoyl-transferase I; CPT II, carnitine-palmitoyl-transferase II; IMM, inner mitochondrial membrane; LCFA, long-chain fatty acids; OMM, outer mitochondrial membrane. (Modified from Lee et al, 2004).

CHAPTER 4

FeTMPyP AS A TREATMENT FOR INFANT INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

CHAPTER 4

FeTMPyP AS A TREATMENT FOR INFANT INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

4.1 BACKGROUND

As previously discussed, intestinal I/R is an important component of the pathogenesis of several life threatening conditions in adults (Schoenberg et al, 1993). In addition, newborns and infants are at risk from the consequences of intestinal I/R, which is involved in the pathogenesis of necrotizing enterocolitis (Eaton et al, 2004), can cause intestinal necrosis following midgut volvulus (Clark, 2000) or intussusception (Fallat, 2000), and can result from splanchnic hypoperfusion during sepsis (Fink, 1993) or after hemorrhage (Moore, 1999). As discussed in Chapter 1, MODS is a well-recognised and potentially lethal complication of paediatric conditions associated with intestinal I/R injury (Morecroft et al, 1994a). In particular, respiratory failure has been shown to be a crucial determinant of early mortality in clinical conditions associated with intestinal reperfusion injury in the neonatal and paediatric age such as NEC and sepsis (Sonntag et al, 1998; Smith et al, 1991; Morecroft et al, 1994a).

Although there has been little work concerning intestinal I/R injury in developing animals, experimental studies in adult animal models have shown that the development of an inflammatory response plays a pivotal role in the pathogenesis of the injury to the intestine and distant organs (Cuzzocrea et al, 2000; Chung et al, 2001). In particular, infiltration of neutrophils and their subsequent action is believed to be responsible for tissue damage not only within the intestine itself (Sisley et al, 1994; Hernandez et al, 1987), but also in distant organs, such as lungs, liver and heart (Schmeling et al, 1989; Simpson et al, 1993; Vinardi et al, 2003; Horie et al, 1996) following intestinal I/R, and neutrophil infiltration correlates with histological damage in human NEC (see Chapter 7). Leukocyte-endothelial cell interactions represent a crucial step in migration of leukocytes from the bloodstream into tissues, which is mediated by several adhesion molecules expressed by activated endothelium (including P-selectin,

ICAM-1 and VCAM-1) in response to the production of interleukins, TNF- α , LPS, histamine, PAF and other soluble mediators (Carlos et al, 1994).

Tissue injury occurs following neutrophil infiltration as a consequence of the release of a wide array of enzymes by activated neutrophils (Moraes et al, 2003). In addition, the production of reactive oxygen species by activated neutrophils (Cuzzocrea et al, 2001b), as well as from xanthine oxidase and other intracellular sources, can lead to a state of oxidative stress once the scavenging potential of endogenous antioxidant systems is overwhelmed (Li et al, 2002), resulting in protein modification, lipid membrane disruption and DNA damage (Tan et al, 1999; Cuzzocrea et al, 2001b).

Of the variety of ROS and RNS generated during oxidative stress, peroxynitrite is thought to be one of the most reactive (see Chapter 1). Peroxynitrite is produced by the spontaneous combination of NO and superoxide released during reperfusion (Beckman et al, 1990; Cuzzocrea et al, 2001b) and it is believed to be responsible for a significant proportion of deleterious effects previously attributed to NO or superoxide alone, including DNA strand scission (Groves et al, 1995; Szabo et al, 1997), lipid peroxidation (Rubbo et al, 1994) and enzyme inactivation (Radi et al, 1994; Fukumoto et al, 2004a; Castro et al, 1994). Peroxynitrite decomposition catalysts are a class of porphyrin-containing compounds that catalyse the decomposition of peroxynitrite to non-radical nitrate and nitrite (Figure 4.1). In particular, 5,10,15,20-tetrakis(*N*-methyl-4'-pyridyl)porphyrinato iron (III) (FeTMPyP, Figure 4.2) has been shown to effectively reduce intestinal injury in experimental endotoxaemia and to improve cerebral I/R injury (Salvemini et al, 2001; Thiagarajan et al, 2004; Thiagarajan et al, 2004). Part of the protective effect of FeTMPyP may be exerted via an interruption of the inflammatory cascade by preventing activation of NF- κ B (Kang et al, 2001). A similar compound, 5,10,15,20-tetrakis (2,4,6-trimethyl-3,5-disulfonatophenyl)-porphyrinato iron (III) (FeTMPS) has been shown to reduce local injury following intestinal I/R in adult rats (Cuzzocrea et al, 2000).

The aim of this study was to characterise intestinal and pulmonary injury in an infant rat model of intestinal I/R, and to evaluate the effects of FeTMPyP as a possible therapeutic agent.

4.2 MATERIALS AND METHODS

4.2.1 Study design

Suckling mixed-sex Sprague-Dawley rats of 11-13 days of age weighing between 20 and 30g were used. The suckling rats were kept with their dams and allowed to suckle freely until anaesthetised. The family groups were kept under standard conditions for food, light and temperature.

Animals were randomly assigned to one of three experimental groups (n=11 per group):

- A) Control Saline: sham operation for 130min with saline infusion
- B) I/R Saline: 40min intestinal ischaemia, followed by 90min reperfusion with saline infusion
- C) I/R FeTMPyP: 40min intestinal ischaemia, followed by 90min reperfusion with FeTMPyP infusion

Core body temperature was monitored throughout the experiment via a rectal probe since induction of anaesthesia, and was maintained within normal values for suckling rats of this age (i.e. between 34 and 36°C) (Bertin et al, 1993) using a heating blanket and lamp.

4.2.2 Surgical Procedure

General anaesthesia was performed by inhalation of isoflurane in an oxygen and nitrous oxide mixture as described in Chapter 2.

Intestinal ischaemia and reperfusion, and sham operation were performed as described in Chapter 2.

Immediately after occlusion of the superior mesenteric artery, the right femoral vessels were exposed through a longitudinal incision, and a 0.16mm polyimide catheter (Cole-Parmer, Vernon Hills, IL, USA) was inserted in the right femoral vein for fluid infusion. Five minutes before the end of ischaemia, rats received either 5ml/kg 0.9% saline (Control Saline group and I/R Saline group), or 30mg/kg FeTMPyP (Calbiochem, Nottingham, UK) in 5ml/kg 0.9% saline (I/R FeTMPyP group) through the femoral line.

One minute before the end of the experiment, all animals were infused with

1,000IU/kg sodium heparin (Sigma-Aldrich Co., Poole, UK) in 5ml/kg 0.9% saline. At the end of the experiment, the thorax was opened through a midline sternotomy, the ascending aorta and the superior vena cava were identified and divided, and animals were sacrificed by exsanguination.

4.2.3 Tissue extraction and metabolite measurement

Immediately after sacrifice, the heart was removed and placed in ice-cold medium for total CPT activity measurement. A blood sample was collected from the thorax, transferred into a heparin tube and centrifuged (3000rpm for 5min at 4°C), and plasma was stored at -70°C until analysis. Small intestine (10-25cm from the ileocaecal valve) and lungs were also removed and stored at -70°C. Terminal ileum (5 to 10cm from the ileocaecal valve) was fixed in 10% formaldehyde for 24 hours and embedded in paraffin.

Myeloperoxidase activity in ileum and lungs was measured by spectrophotometry. Levels of malondialdehyde in terminal ileum, lungs and heart were determined by HPLC. The concentration of reduced and oxidised glutathione in ileum was measured by HPLC. Plasma concentration of nitrate plus nitrite, reflecting systemic production of nitric oxide, was quantified by HPLC. Total carnitine palmitoyl transferase activity in the heart was measured by radiochemical assay. Assays for MDA, glutathione, nitrate plus nitrite, and total CPT activity were performed as described in Chapter 2.

Haematoxylin and eosin staining, and immunostaining for P-Selectin, ICAM-1, and VCAM-1 were performed on 5µm sections from paraffin embedded samples of terminal ileum as described in Chapter 2. The degree of histological injury and the level of expression of each adhesion molecule were assessed semi-quantitatively as specified in Chapter 2.

4.2.4 Statistical analysis

Continuous variables are expressed as mean \pm SEM and compared by one-way ANOVA with Tukey post hoc test. Data referring to discrete variables are expressed as median [interquartile range], and Kruskal-Wallis test was used for group comparison. Linear regression was used to evaluate the correlation between

levels of MDA and glutathione in the ileum; and Spearman R to assess the relationships between histological injury, P-Selectin and ICAM-1 expression, and MPO activity. Results showing p values <0.05 were considered significant.

4.3 RESULTS

4.3.1 *Histological injury*

Ileal histology was normal in all control animals (Figure 4.3, 4.4 A). All animals subjected to intestinal I/R and saline infusion showed evidence of microscopical tissue damage: the degree of histological injury varied from presence of mucosal slough at villous tips (Grade 2) to complete disintegration of the *lamina propria* (Grade 5), with the majority of samples showing denuded villi with exposed but still intact *lamina propria* (Grade 4, Figure 4.3, 4.4 B). Rats undergoing intestinal I/R and FeTMPyP administration (Figure 4.3, 4.4 C) showed overall a somehow milder degree of histological damage compared to the I/R plus saline group: in few animals the ileum appeared microscopically normal (Grade 1), and in no one there was evidence of destruction of the *lamina propria*. Congestion of blood vessels and evidence of haemorrhage also developed as a consequence of intestinal reperfusion, which were more frequent and severe in rats receiving saline than in those treated with the scavenger. However, statistical analysis of histological injury according to the grading system used showed no difference between the I/R plus saline and I/R plus FeTMPyP groups (Figure 4.3).

4.3.2 *P-Selectin expression*

Expression of P-Selectin on blood vessels was weak in most specimens from control rats (Figure 4.5, Figure 4.6 A). Intestinal I/R induced a significant increase in the expression of P-Selectin, most animals showing moderate to strong staining (Figure 4.5). Vessels expressing P-Selectin were evenly distributed within the specimens, and staining was particularly intense on medium-sized vessels of the submucosa (Figure 4.6 B). However, expression of P-Selectin on I/R rats receiving FeTMPyP showed only a non-significant trend towards increase as compared to controls (Figure 4.5).

A strong positive correlation was found between intensity of expression of P-Selectin on blood vessels and degree of histological injury in the ileum ($r=0.580$; $p=0.0004$). In addition, the degree of P-Selectin expression in the ileum also correlated with the extent of intestinal neutrophil infiltration as assessed by MPO activity ($r=0.554$; $p=0.0008$).

4.3.3 ICAM-1 expression

Mild expression of ICAM-1, particularly on medium-size vessels of the submucosa, was evident in the ileum of most rats from the control group, reflecting constitutive expression of ICAM-1 (Figure 4.8 A). However, ICAM-1 expression was increased following intestinal I/R in animals receiving both saline and FeTMPyP (Figure 4.7), which showed a more diffuse and intense staining on medium-sized vessels in the submucosa (Figure 4.8 B). In addition, in the latter two groups considerable expression of ICAM-1 was observed also on the microcirculation, particularly in the submucosa, which was virtually absent in specimens from control rats.

4.3.4 VCAM-1 expression

Expression of VCAM-1 was variable in ileum specimens from control animals (Figure 4.9). In most specimens, VCAM-1 was expressed in a patchy pattern, and staining was present not only on medium size vessels, but also on the microcirculation in the submucosa (Figure 4.10 A, 4.10 B). Rats undergoing intestinal I/R, both with and without FeTMPyP, did not show any quantitative increase in VCAM-1 expression (Figure 4.9), and the type and location of positively stained vessels was also similar to that of controls (Figure 4.10 C).

4.3.5 Neutrophil infiltration

Following intestinal I/R, neutrophil infiltration was greatly increased not only in the ileum (Figure 4.11 A), but also in the lungs (Figure 4.11 B), as indicated by myeloperoxidase activity. FeTMPyP administration prevented the infiltration of neutrophils in the ileum: I/R animals receiving the scavenger showed levels of MPO comparable to controls, and significantly lower than I/R animals

resuscitated with saline (Figure 4.11 A). However, FeTMPyP did not affect the degree of neutrophil infiltration in the lungs, and pulmonary MPO activity was similar in I/R animals receiving either the scavenger or saline (Figure 4.11 B).

4.3.6 Lipid peroxidation

Intestinal ischaemia and reperfusion was also associated with an increase in lipid peroxidation in both intestine and lungs in animals that received infusion of saline compared to controls, but this effect was prevented by administration of FeTMPyP (Figure 4.12 A, 4.12 B). Interestingly, no differences in cardiac levels of malondialdehyde were observed in the heart following intestinal reperfusion (Figure 4.12 C). Quantification of MDA levels in plasma was attempted, however no reliable measurement could be obtained due to interfering peaks possibly generated by FeTMPyP in solution.

4.3.7 Glutathione content

A profound depletion in intestinal total glutathione levels was observed following intestinal I/R in rats infused with saline only as compared to controls (Figure 4.13 C). This effect appeared to be due to a decrease in both reduced (Figure 4.13 A) and oxidised glutathione (Figure 4.13 B). Administration of FeTMPyP partially prevented the decrease in glutathione concentration caused by intestinal I/R: concentrations of total and oxidised glutathione were significantly higher in I/R animals treated with the scavenger compared to those receiving saline, and a trend towards increase was seen in reduced glutathione. However, no differences were seen in glutathione redox state in the ileum (Figure 4.13 D), indicating that cell redox potential was unaffected by intestinal reperfusion injury.

Interestingly, intestinal levels of total glutathione negatively correlated with MDA concentration ($r^2=0.195$; $p=0.010$), suggesting an association between a decrease in antioxidant potential and development of lipid peroxidation.

4.3.8 Systemic nitric oxide production

An increase in nitric oxide production was observed as a consequence of intestinal I/R in animals receiving saline infusion with respect to controls (Figure

4.14). Administration of the peroxynitrite decomposition catalyst effectively reduced nitric oxide synthesis, and levels of nitrate plus nitrite in I/R rats receiving FeTMPyP were comparable to controls.

4.3.9 *Total carnitine palmitoyl transferase activity in the heart*

Values of total CPT activity were similar in all experimental groups, suggesting influx of fatty acids into the mitochondria was unaffected in this model of intestinal I/R (Figure 4.15)

4.4 DISCUSSION

I/R of the intestine is known to cause a cascade of local (intestinal) and distal (liver, lung, heart, kidney) damage and organ failure which may be triggered by neutrophil infiltration with subsequent release of proteolytic enzymes and reactive oxygen and nitrogen species. Therapeutic strategies that have been shown to prevent organ damage in intestinal I/R appear to exert their effects via this neutrophil infiltration - free radical - organ damage axis. Examples of this are moderate hypothermia (See Chapter 3 and Vinardi et al, 2003), agents such as anti-adhesion molecule antibodies or other methods to deplete neutrophils (Hernandez et al, 1987; Horie et al, 1996; Sisley et al, 1994) and scavengers of ROS and RNS (Cuzzocrea et al, 2000; Cuzzocrea et al, 2001a; Naito et al, 2004). However, many ROS and RNS have important physiological roles such as maintenance of vascular tone (nitric oxide) and bacterial killing (superoxide) so that deleterious as well as beneficial effects could result from their administration. Peroxynitrite, on the other hand, is a reactive species with no well-defined physiological function so could potentially be a more useful valid pharmacological target.

The compound which I used in this study is one of a large group of transition metal based agents which scavenge a variety of reactive nitrogen and oxygen species, and the dose chosen (30mg/kg) showed to be effective in a model of endotoxin-induced intestinal injury (Salvemini et al, 1999). Some of this group of compounds are already in use in clinical trials for relief of dermatological pain. Although FeTMPyP is very specific for scavenging peroxynitrite, it appears to

interrupt the cycle of adhesion molecule expression, neutrophil infiltration, antioxidant depletion, free radical production and histological damage. This finding supports a role for peroxynitrite in initiating this cascade. My results in the current study in infant rats, using FeTMPyP, are broadly similar to those of Cuzzocrea et al in adult rat intestinal I/R injury, using a related peroxynitrite scavenger, FeTMPS (Cuzzocrea et al, 2000). There are, however, some differences between these studies, in addition to the use of infant rats and a different peroxynitrite decomposition catalyst. Cuzzocrea et al administered the scavenger after only 15min of ischaemia (30min before reperfusion), whereas I administered the scavenger 5min before reperfusion (after 35min of ischaemia). Potential clinical use of peroxynitrite decomposition catalysts would probably involve administration close to the initiation, or even after commencement, of reperfusion (e.g. surgery for midgut volvulus). Interestingly, in a rat middle cerebral artery occlusion model of stroke, FeTMPyP was protective even when given 6 hours after the acute insult (Thiyagarajan et al, 2004), suggesting that administration could be effective even when applied later, although I did not test this in my model.

In the present study, I additionally showed a protective effect of FeTMPyP against lipid peroxidation in the lungs, which is important as multiple organ failure is an important consequence of diseases involving intestinal reperfusion injury.

Although peroxynitrite scavengers decreased both ileal P-selectin expression and neutrophil infiltration in my study and that of Cuzzocrea, in their study, the peroxynitrite scavenger prevented up-regulation of ICAM expression, whereas in my study, it had no effect. Findings from the current study suggest that prevention of P-selectin expression, and therefore of early neutrophil rolling, is sufficient to attenuate neutrophil infiltration, even when the elevated ICAM-1 expression, which mediates later firm adhesion, is unaltered. In other studies using peroxynitrite scavengers, it has been shown that removal of peroxynitrite can prevent plasma leakage into the intestinal tract in endotoxaemia (Salvemini et al, 1999). Other similar effects have been observed using structurally-related superoxide dismutase mimetics (Salvemini et al, 1999; Cuzzocrea et al, 2001a) which, by scavenging superoxide, would also prevent peroxynitrite formation, as

peroxynitrite can be physiologically generated by the reaction of superoxide with nitric oxide.

In the current study, total carnitine palmitoyl transferase (CPT) activity was not impaired following 40min mesenteric ischaemia and 90min reperfusion. The shorter time frame used in this study as compared to the experiment described in Chapter 3 could account for this difference, and animals could have been sacrificed before potential alterations of CPT activity (e.g. tyrosine nitration of CPT I by peroxynitrite) ensue. This is consistent with previous studies from my group, where alterations in cardiac CPT I activity were observed at 2 hours following intraperitoneal injection of LPS in infant rats (Fukumoto et al, 2002a; Fukumoto et al, 2002b).

In the current experiment, as FeTMPyP was administered systemically, it was impossible to determine whether the effects of FeTMPyP administration on distal organs and systemic measures are secondarily mediated via the effect of FeTMPyP on the intestine, or whether FeTMPyP is also acting systemically. Interestingly, FeTMPyP decreased neutrophil infiltration and lipid peroxidation in the intestine, but lipid peroxidation in the lungs was decreased despite neutrophil infiltration in the lungs remaining elevated, suggesting that FeTMPyP could be acting locally in the lung to prevent peroxynitrite-initiated lipid peroxidation. However, FeTMPyP catalyses the decomposition of peroxynitrite to nitrate, so that if the effects of FeTMPyP were systemic, one could expect an increase in plasma nitrate plus nitrite, rather than the decrease we observed in the current study. The observed decrease suggests that FeTMPyP may be primarily effective locally in the intestine, thus preventing a systemic inflammatory response. In addition, this prevention of lipid peroxidation in the ileum by FeTMPyP seems to be unrelated to major antioxidant systems, since a similar decrease in both reduced, oxidized, and total glutathione concentrations was observed following mesenteric reperfusion in animals receiving saline and in those treated with FeTMPyP. Further experiments would be required to determine the mechanism of action.

Although quantification of tissue or plasma nitrotyrosine levels would have contributed to support the role of peroxynitrite-induced nitrosative stress in this model of intestinal I/R, the partial protection achieved by FeTMPyP in this study

can be entirely attributed to the decomposition of peroxynitrite (Cuzzocrea et al, 2001b), since this compound does not exert catalase or superoxide dismutase activity (Salvemini et al, 1999). In fact, immunostaining for nitrotyrosine was attempted on paraffin-embedded sections of small intestine from experimental animals, however no reliable staining could be obtained despite several different modalities of antigen retrieval.

Various compounds of this class of porphyrin derivatives have been synthesised, and a closely related compound, M-40403, which is specific for scavenging superoxide, is currently being tested in phase II clinical trials for inflammatory conditions and pain (Di Napoli et al, 2005). M-40403 contains Mn rather than Fe at the catalytic centre, as metabolism of Fe-containing porphyrins could potentially lead to the generation of harmful Fe-centered radicals. However, all the metal-centered porphyrin compounds are very stable and there are no reports of pro-oxidant activities “in vivo”. A further potential problem with antioxidant therapy is interference with the bacterial killing process and increased susceptibility to infection. Peroxynitrite is toxic to bacteria and may be involved with bacterial killing following phagocytosis (Fang, 2004; Zhu et al, 1992). In addition, it may have other physiological roles (Ferdinandy, 2006), so that scavenging peroxynitrite may have other effects in addition to prevention of the inflammatory process.

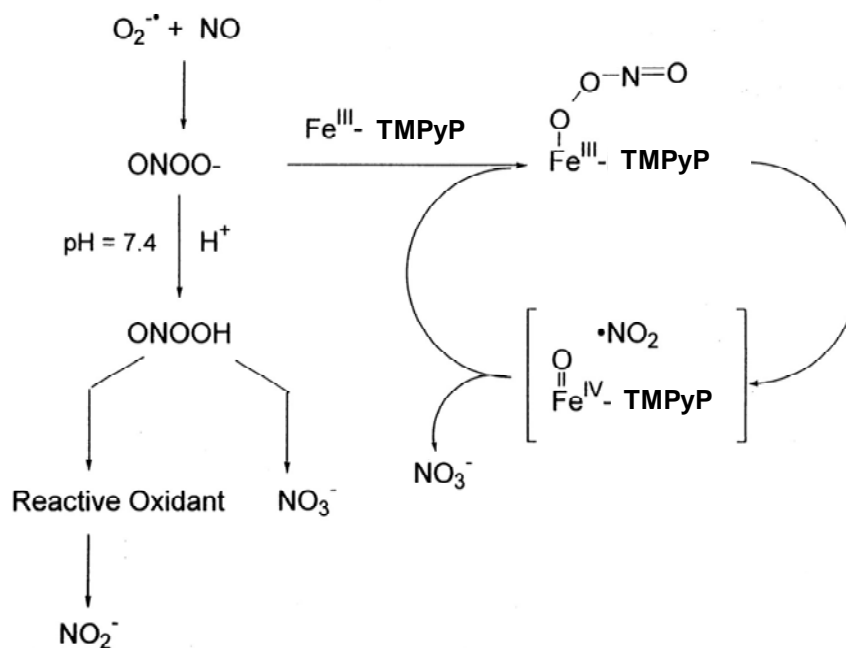
5.5 CONCLUSIONS

Superior mesenteric artery ischaemia and reperfusion was characterised by significant alterations not only in the gut, but also in distant organs in my infant rat model. Upregulation of endothelial adhesion molecules (P-Selectin and ICAM-1) was detected in the distal ileum, which was accompanied by increased inflammatory cell infiltration and development of oxidative stress, ultimately resulting in tissue injury and disruption of mucosal architecture. Recruitment of neutrophils and development of lipid peroxidation of biological membranes were observed in the lungs, together with an increased in nitrosative stress at a systemic level.

Systemic administration of FeTMPyP at reperfusion exerted a protective effect on the gut, reducing neutrophil infiltration and lipid peroxidation in the intestine, possibly via inhibition of P-Selectin expression on vascular endothelium, although it did not reduce the extent of histological injury. The use of this peroxynitrite decomposition catalyst afforded protection also to distant organs, preventing the development of oxidative stress in the lungs and inhibiting systemic overproduction of nitric oxide.

Peroxynitrite decomposition scavengers have been shown to be effective in a variety of models, and are promising candidates for clinical trials to prevent systemic inflammatory response syndrome, especially given the apparently relative broad window of opportunity for their use. However, careful clinical studies are required to determine whether there is increased susceptibility to infection and/or other side effects.

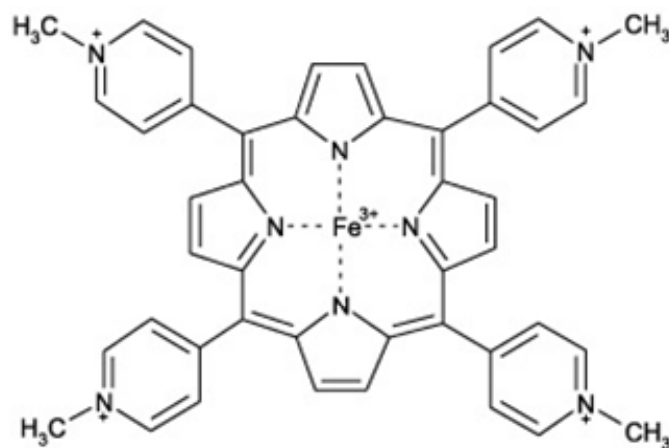
Figure 4.1. Proposed mechanism of action for peroxynitrite decomposition catalyst FeTMPyP (Salvemini et al, 1998).



Schematic representation of the proposed chemical mechanism for peroxynitrite decomposition catalyst FeTMPyP. The predominant product of FeTMPyP mediated decomposition of peroxynitrite is nitrate (>90%), with a minor amount of nitrite also formed (approximately 7%). Spontaneous reaction of peroxynitrite with H^+ , on the contrary, produces both nitrate (approximately 65%) and nitrite (approximately 35%).

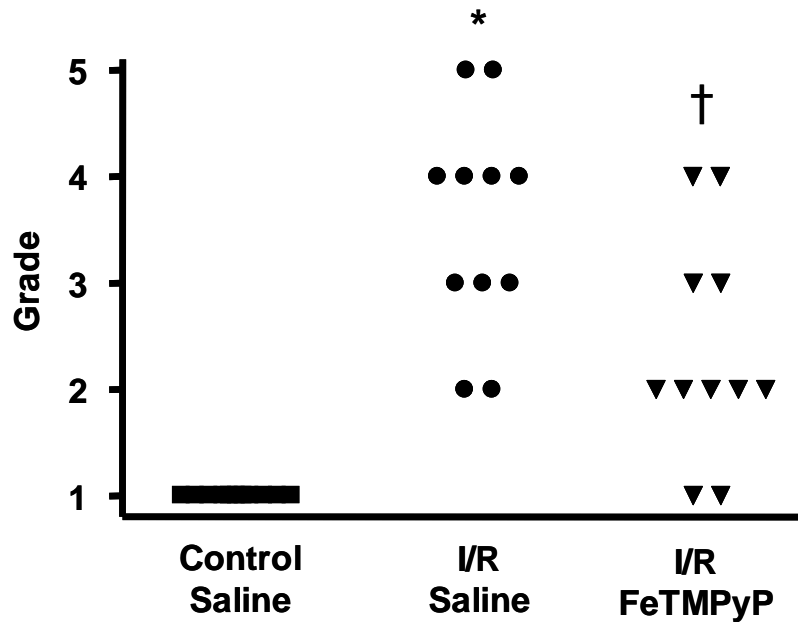
(Modified from Salvemini et al, 1998).

Figure 4.2. Molecular structure of peroxynitrite decomposition catalyst FeTMPyP.



Molecular structure of peroxyinitrite decomposition catalyst FeTMPyP (5,10,15,20-tetrakis(N-methyl-4'-pyridyl)porphyrinato iron (III)), containing an iron molecule in a porphyrin ring.

Figure 4.3. Histological injury in ileum.

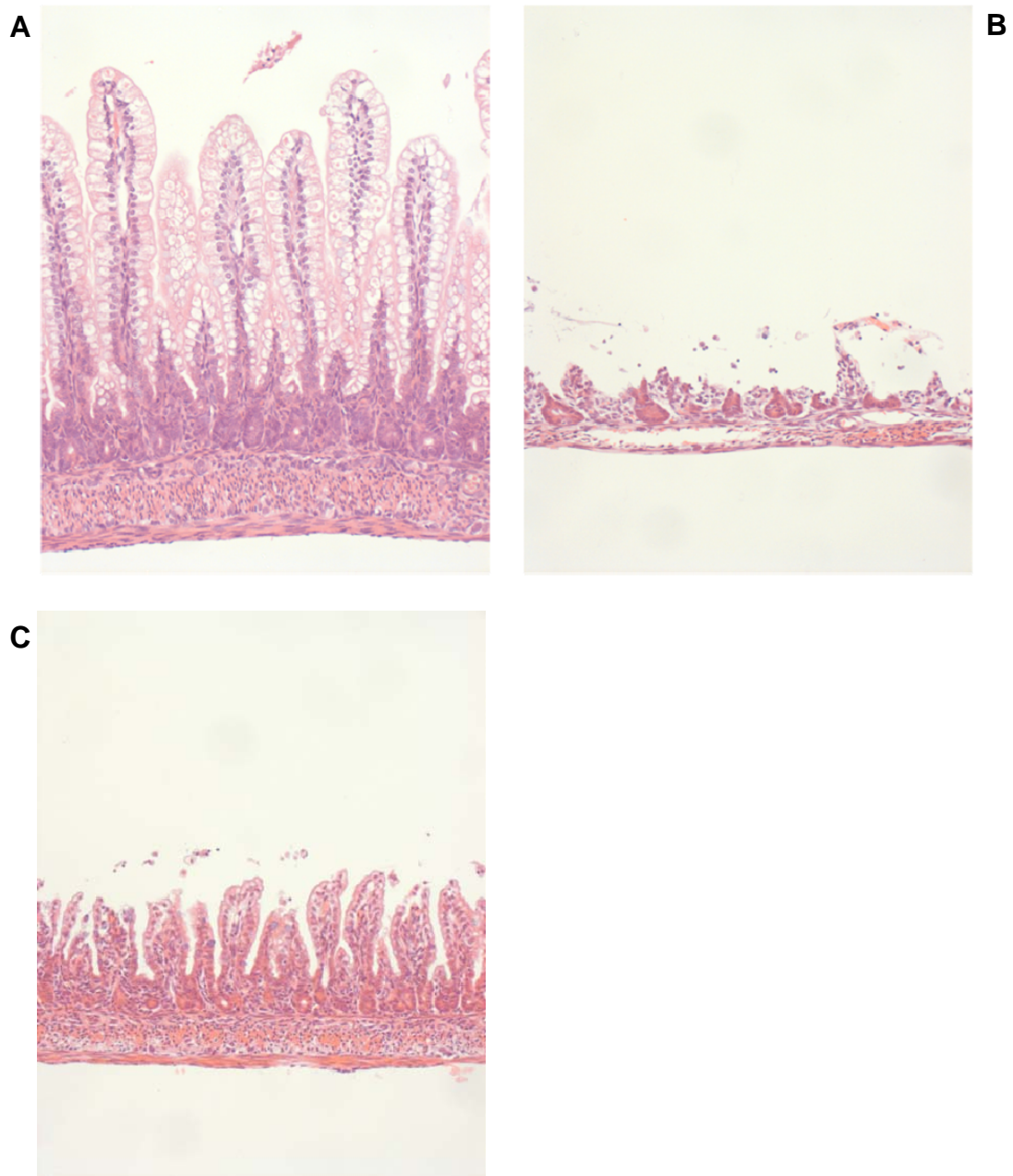


Grade of histological injury in the ileum (5=maximum, 1=minimum). Animals (n=11 per group) underwent sham operation with saline infusion (Control Saline) or intestinal ischaemia and reperfusion with infusion of either saline (I/R Saline) or FeTMPyP solution (I/R FeTMPyP). Results were compared using Kruskal-Wallis with Dunn's multiple comparison test.

* I/R Saline: $P < 0.001$ vs. Control Saline.

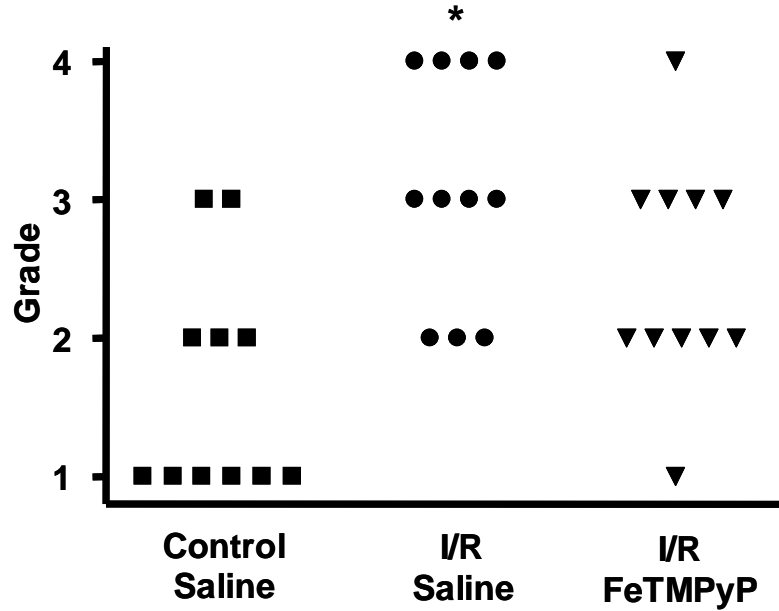
† I/R FeTMPyP: $P < 0.05$ vs. Control Saline.

Figure 4.4. Histological appearance of terminal ileum.



Specimens of terminal ileum were stained with haematoxylin and eosin, and are from: Control Saline [A]; I/R Saline [B]; I/R FeTMPyP [C]. Magnification is x100.

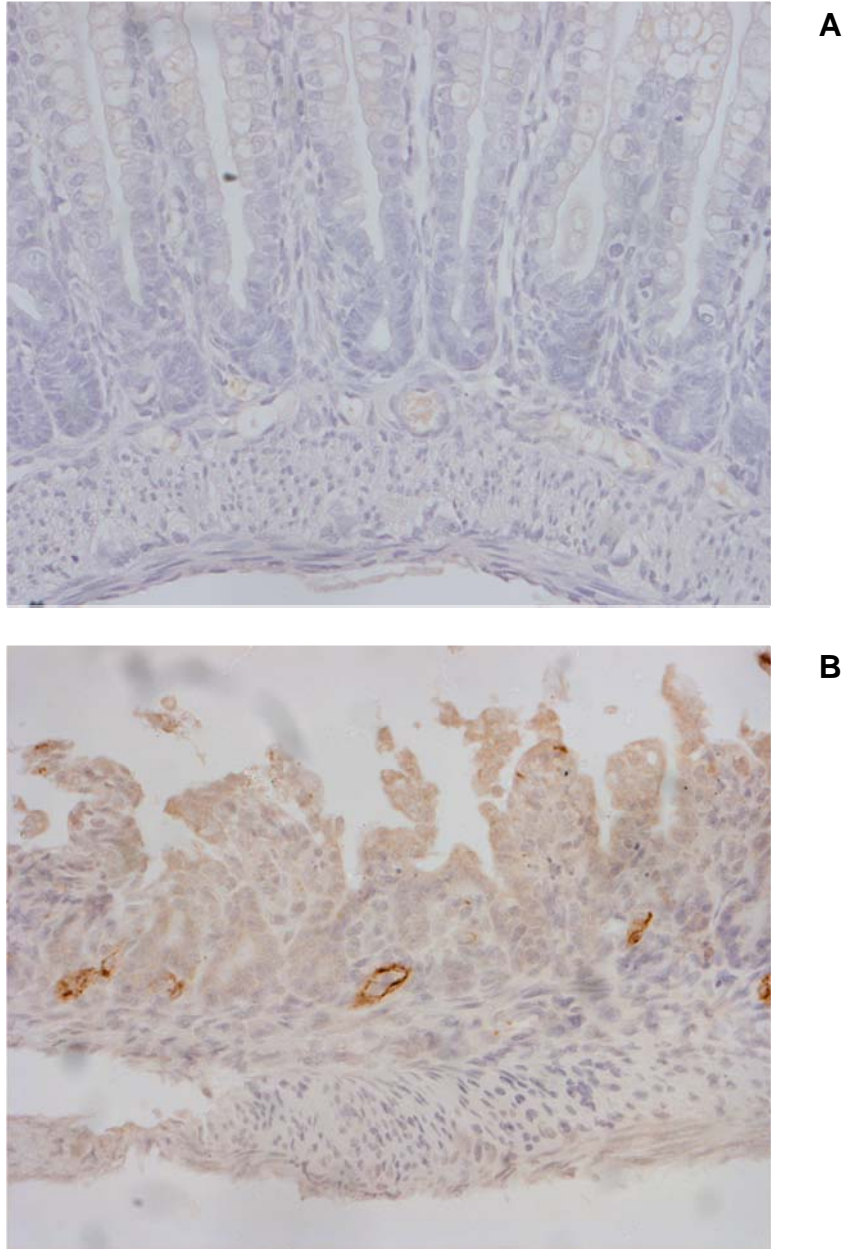
Figure 4.5. P-Selectin expression in ileum.



Grade of P-Selectin expression the ileum (4=maximum, 1=minimum). Animals (n=11 per group) underwent sham operation with saline infusion (Control Saline) or intestinal ischaemia and reperfusion with infusion of either saline (I/R Saline) or FeTMPyP solution (I/R FeTMPyP). Results were compared using Kruskal-Wallis with Dunn's multiple comparison test.

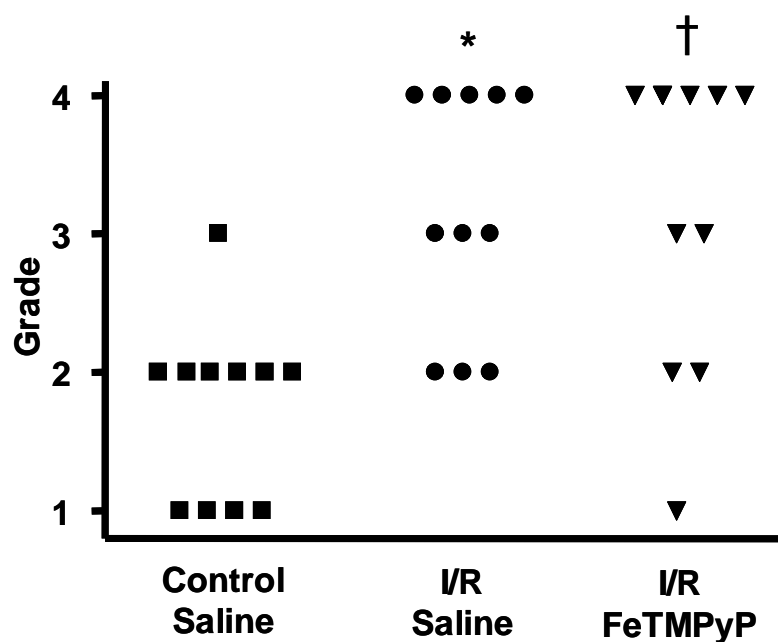
* I/R Saline: $P < 0.01$ vs. Control Saline.

Figure 4.6. Histological appearance of P-Selectin expression in ileum.



Specimens of terminal ileum were immunostained for P-Selectin and counterstained with haematoxylin as described in Chapter 2. Specimens are from: Control Saline [A]; I/R Saline [B]. Magnification is x400.

Figure 4.7. ICAM-1 expression in ileum.

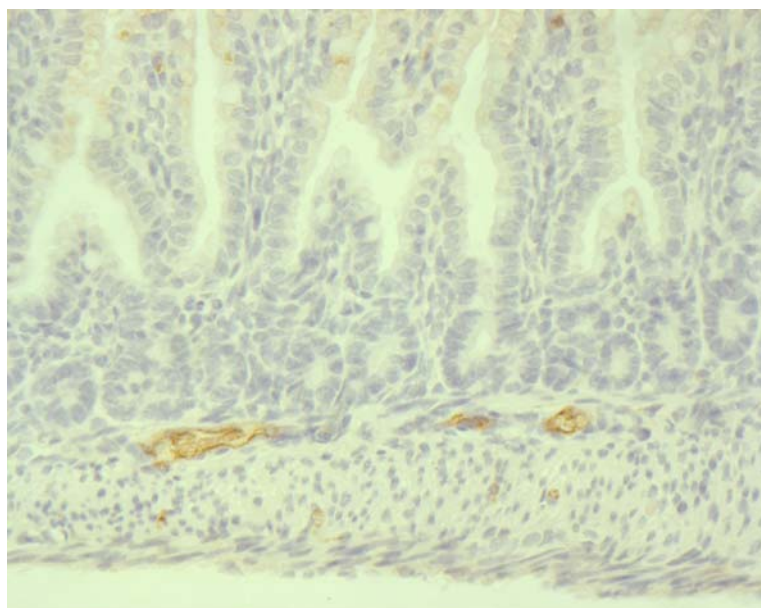


Grade of ICAM-1 expression the ileum (4=maximum, 1=minimum). Animals (n=11 per group) underwent sham operation with saline infusion (Control Saline) or intestinal ischaemia and reperfusion with infusion of either saline (I/R Saline) or FeTMPyP solution (I/R FeTMPyP). Results were compared using Kruskal-Wallis with Dunn's multiple comparison test.

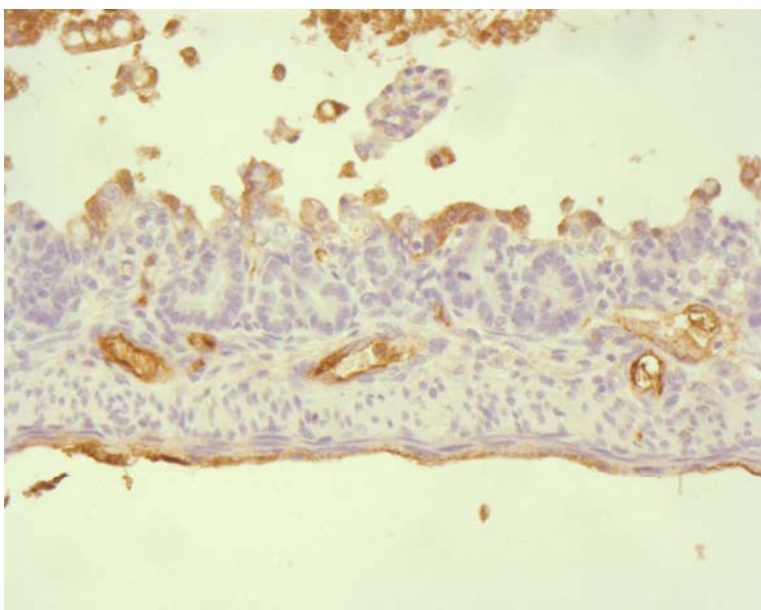
* I/R Saline: $P < 0.01$ vs. Control Saline.

† I/R FeTMPyP: $P < 0.01$ vs. Control Saline.

Figure 4.8. Histological appearance of ICAM-1 expression in ileum.



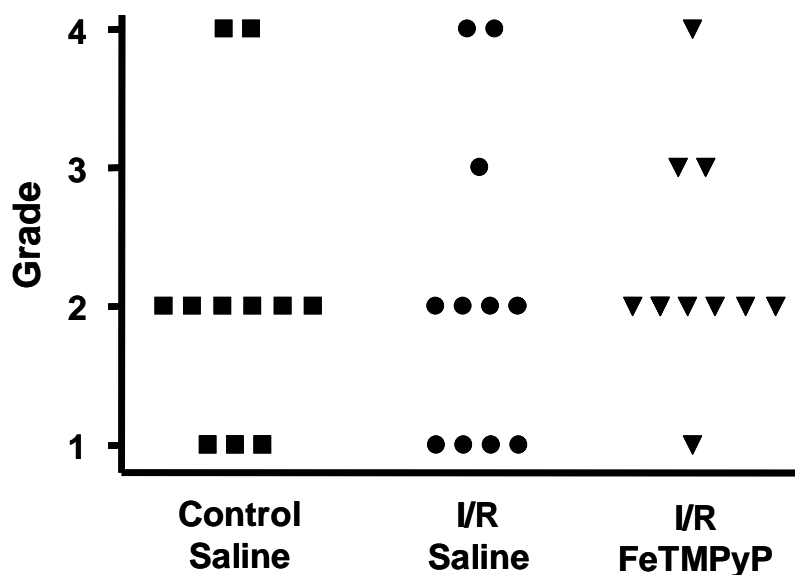
A



B

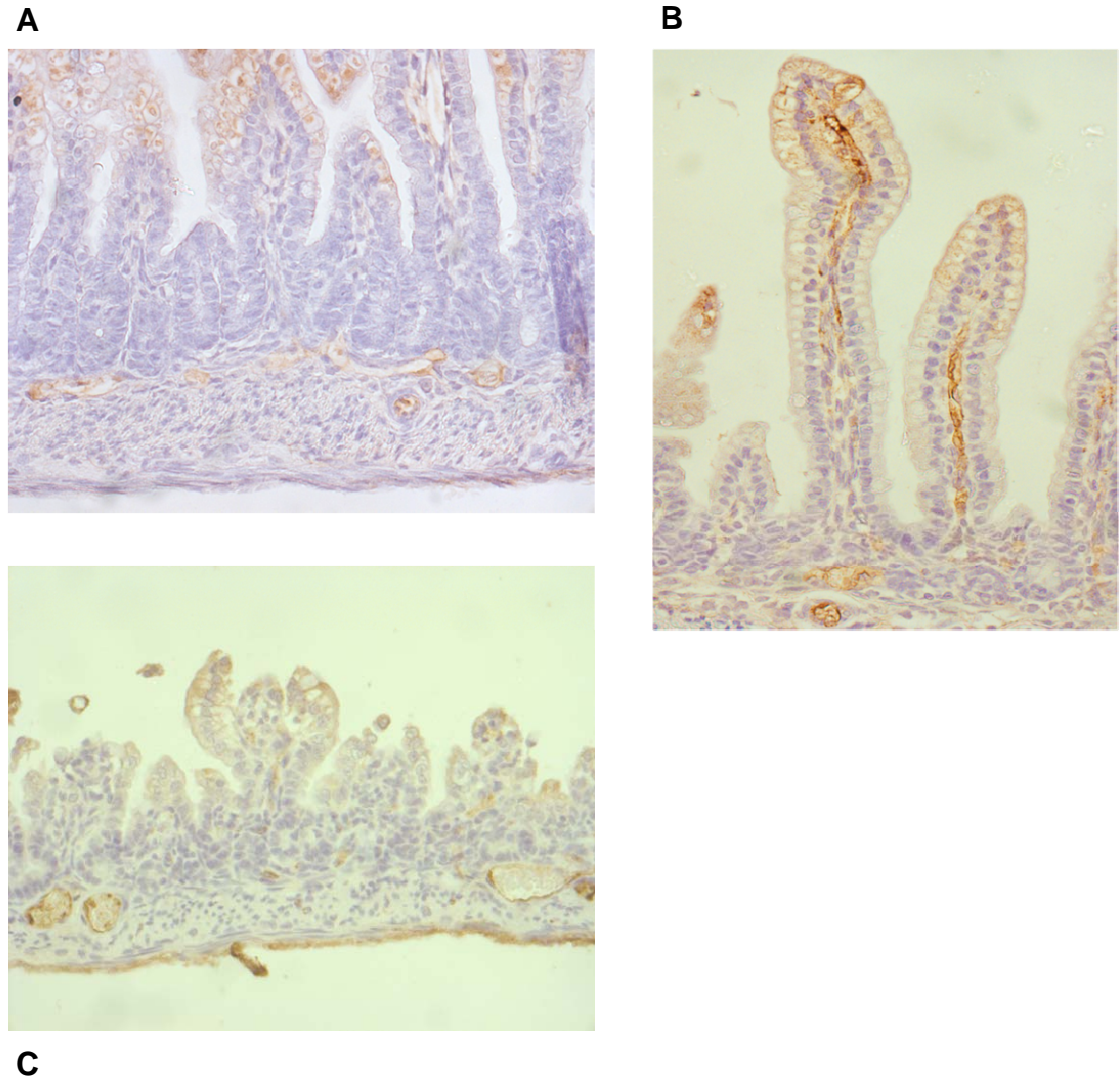
Specimens of terminal ileum were immunostained for ICAM-1 and counterstained with haematoxylin as described in Chapter 2. Specimens are from: Control Saline [A]; I/R Saline [B]. Magnification is x400.

Figure 4.9. VCAM-1 expression in ileum.



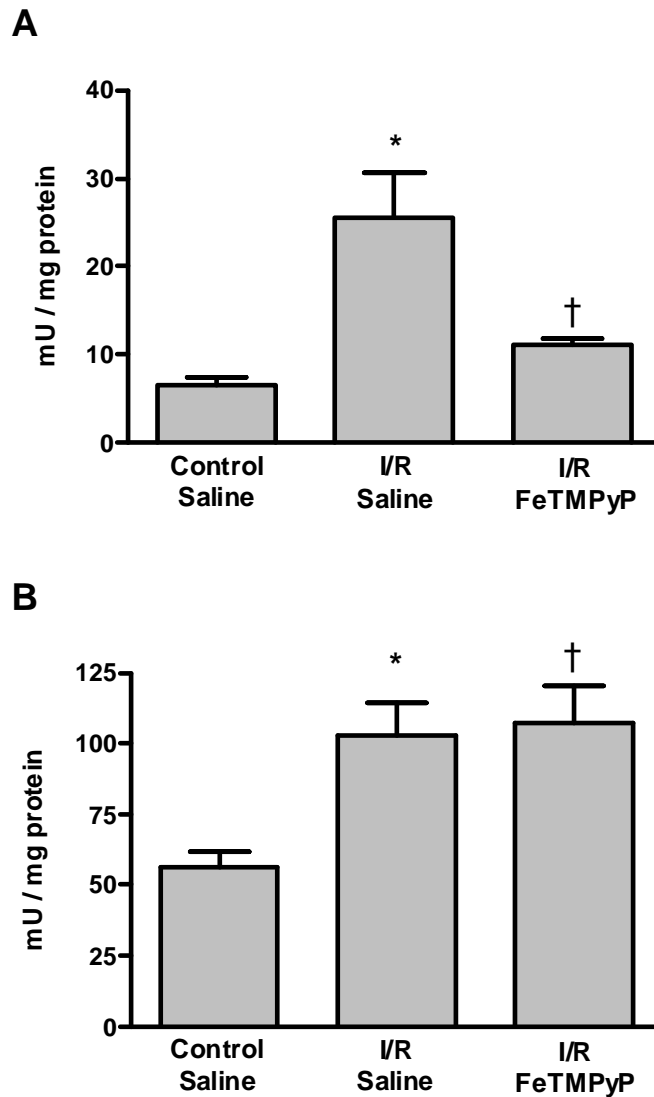
Grade of VCAM-1 expression the ileum (4=maximum, 1=minimum). Animals (n=11 per group) underwent sham operation with saline infusion (Control Saline) or intestinal ischaemia and reperfusion with infusion of either saline (I/R Saline) or FeTMPyP solution (I/R FeTMPyP). Results were compared using Kruskal-Wallis with Dunn's multiple comparison test.

Figure 4.10. Histological appearance of VCAM-1 expression in ileum.



Specimens of terminal ileum were immunostained for VCAM-1 and counterstained with haematoxylin as described in Chapter 2. Specimens are from: Control Saline [A,B]; I/R Saline [C]. Magnification is x400.

Figure 4.11. Neutrophil infiltration in ileum and lungs.



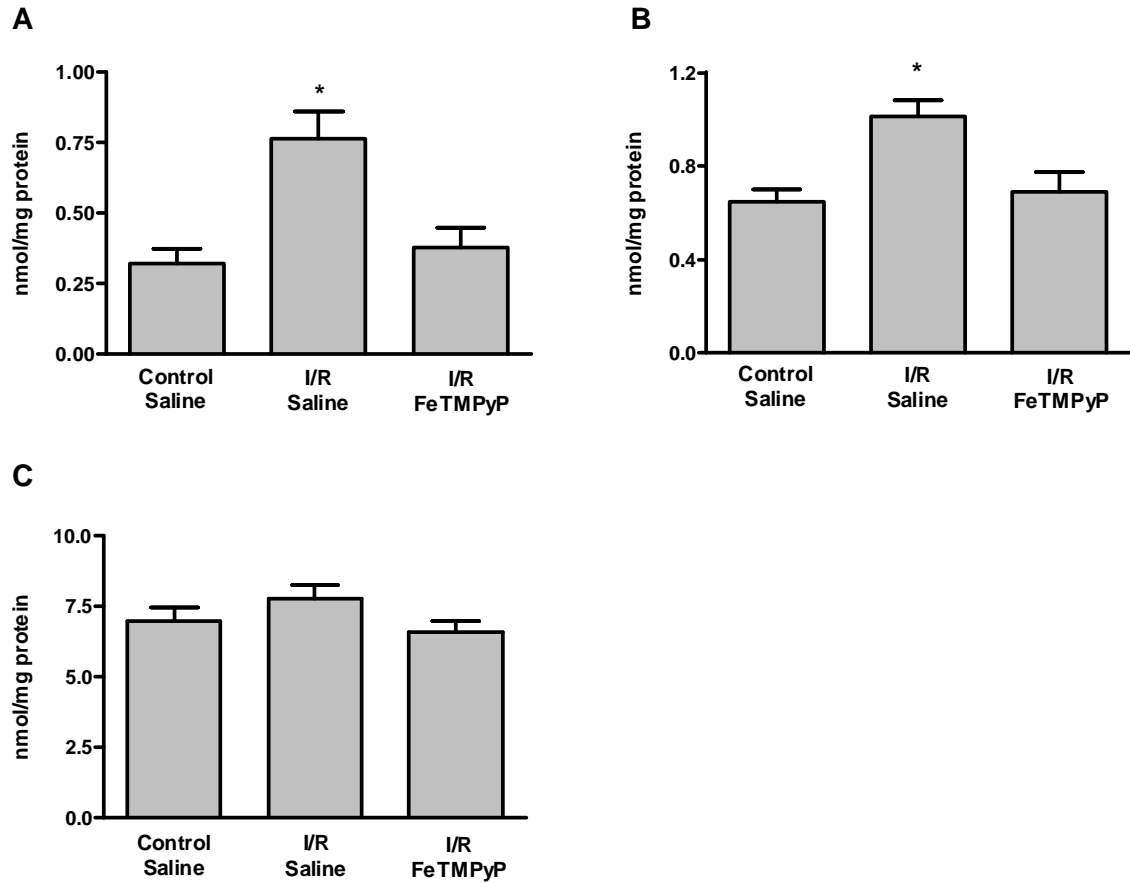
Neutrophil infiltration, assessed as myeloperoxidase activity (MPO) in ileum [A], and lungs [B]. Animals (n=11 per group) underwent sham operation with saline infusion (Control Saline) or intestinal ischaemia and reperfusion with infusion of either saline (I/R Saline) or FeTMPyP solution (I/R FeTMPyP). Data are expressed as nmol/mg protein and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Saline: $P < 0.001$ vs. Control Saline; $P = 0.006$ vs. I/R FeTMPyP.

† I/R Saline: $P = 0.009$ vs. Control Saline.

[B]: * I/R FeTMPyP: $P = 0.004$ vs. Control Saline.

Figure 4.12. Malondialdehyde concentration in ileum, lungs and heart.

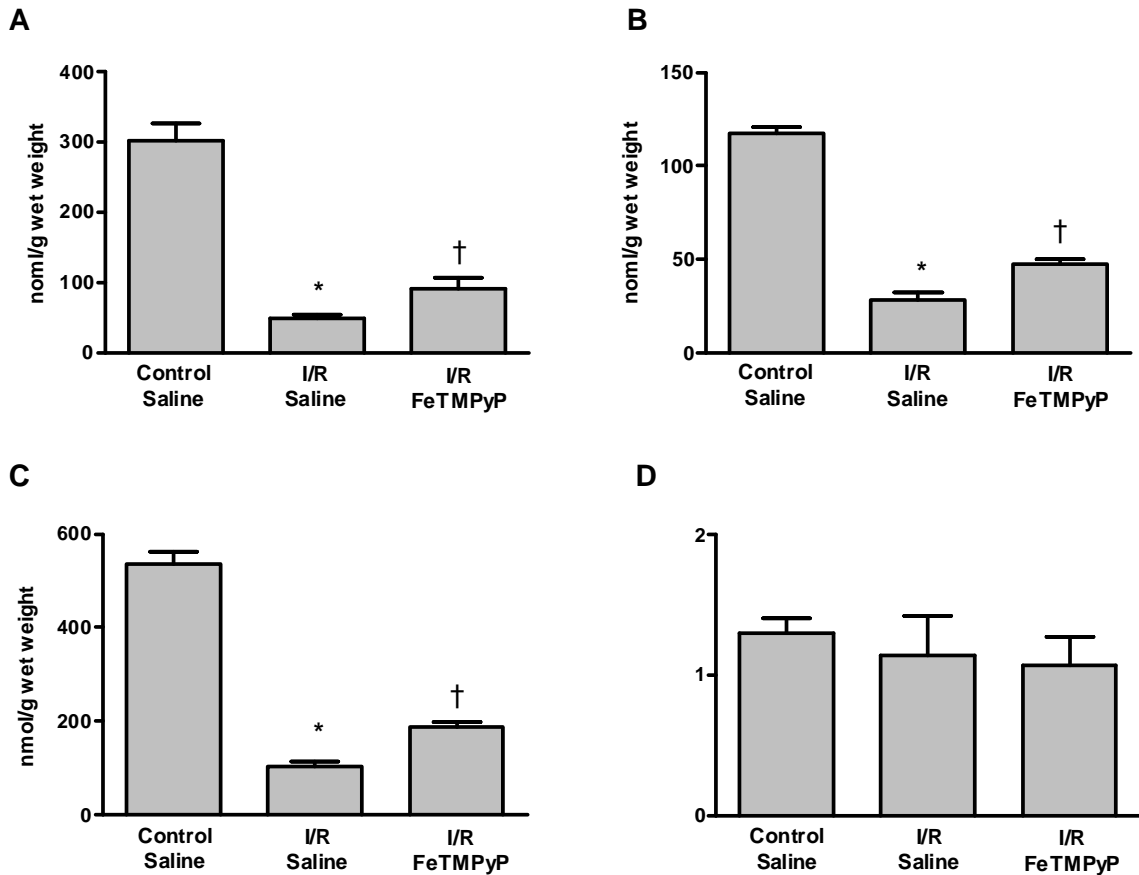


Concentration of malondialdehyde (MDA) in ileum [A], lungs [B], and heart [C]. Animals (n=11 per group) underwent sham operation with saline infusion (Control Saline) or intestinal ischaemia and reperfusion with infusion of either saline (I/R Saline) or FeTMPyP solution (I/R FeTMPyP). Data are expressed as nmol/mg protein and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Saline: P = 0.001 vs. Control Saline; P = 0.003 vs. I/R FeTMPyP.

[B]: * I/R Saline: P = 0.002 vs. Control Saline; P = 0.007 vs. I/R FeTMPyP.

Figure 4.13. Glutathione concentration in ileum.



Concentration of reduced (GSH) [A], oxidised (GSSG) [B], and total (calculated as $GSH + 2 \times GSSG$) [C] glutathione, and glutathione redox state (calculated as $GSH/2 \times GSSG$) [D] in ileum. Animals ($n=11$ per group) underwent sham operation with saline infusion (Control Saline) or intestinal ischaemia and reperfusion with infusion of either saline (I/R Saline) or FeTMPyP solution (I/R FeTMPyP). Concentrations of GSH, GSSG and total glutathione are expressed as nmol/g wet weight. Results are reported as mean \pm SEM; one-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Saline: $P < 0.001$ vs. Control Saline.

† I/R FeTMPyP: $P < 0.001$ vs. Control Saline.

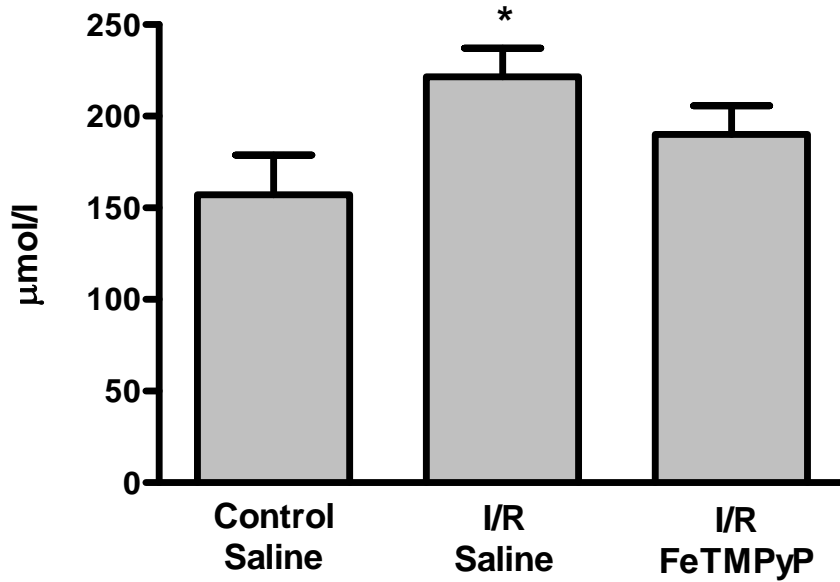
[B]: * I/R Saline: $P < 0.001$ vs. Control Saline.

† I/R FeTMPyP: $P < 0.001$ vs. Control Saline; $P = 0.001$ vs. I/R Saline.

[C]: * I/R Saline: $P < 0.001$ vs. Control Saline.

† I/R FeTMPyP: $P < 0.001$ vs. Control Saline; $P = 0.007$ vs. I/R Saline.

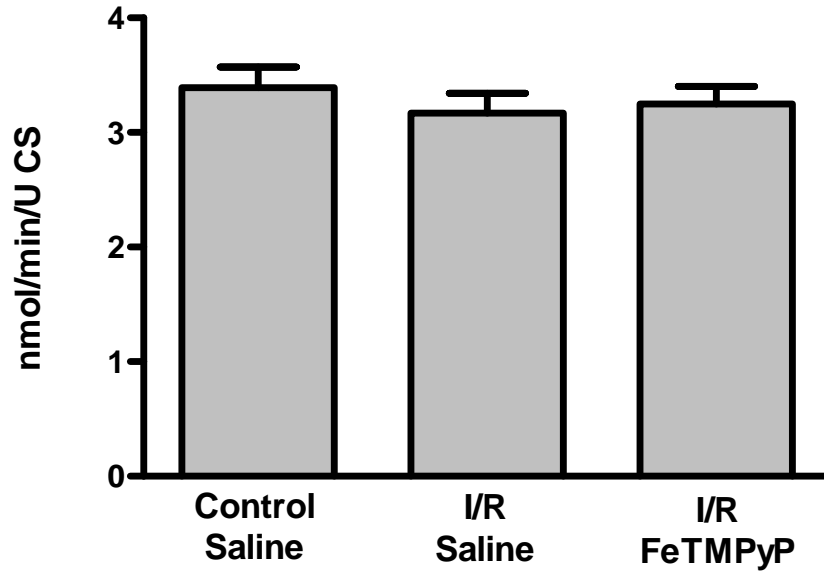
Figure 4.14. Nitrate plus nitrite concentration in plasma.



Systemic production of nitric oxide measured as concentration of nitrate plus nitrite in plasma. Animals (n=11 per group) underwent sham operation with saline infusion (Control Saline) or intestinal ischaemia and reperfusion with infusion of either saline (I/R Saline) or FeTMPyP solution (I/R FeTMPyP). Data are expressed as μmol/l and results are reported as mean ± SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

* I/R Saline: P = 0.041 vs. Control Saline.

Figure 4.15. Total carnitine palmitoyl transferase activity in the heart.



Total carnitine palmitoyl transferase (CPT) activity in the heart Animals (n=11 per group) underwent sham operation with saline infusion (Control Saline) or intestinal ischaemia and reperfusion with infusion of either saline (I/R Saline) or FeTMPyP solution (I/R FeTMPyP). Data are expressed as mmol/min/U citrate synthase (CS) and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

CHAPTER 5

MODERATE HYPOTHERMIA AS A RESCUE TREATMENT FOR ADULT INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

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MODERATE HYPOTHERMIA AS A RESCUE TREATMENT FOR ADULT INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

5.1 BACKGROUND

Therapeutic hypothermia is beneficial in animal models of cerebral (Kollmar et al, 2002), cardiac (Hale et al, 2003), hepatic (Choi et al, 2005) and renal (Zager et al, 1989) ischaemia, and clinical evidence suggests that rescue hypothermia can improve outcome after cardiac arrest (The Hypothermia after Cardiac Arrest Study Group, 2002; Bernard et al, 2002) and neonatal hypoxic-ischaemic encephalopathy (Shankaran et al, 2005; Gluckman et al, 2005). Experimental studies have shown that moderate hypothermia, when applied throughout intestinal I/R, can prevent mortality (Vejchapipat et al, 2001; Kalia et al, 2002b). This hypothermic protection is associated not only with a reduction in intestinal injury (Hassoun et al, 2002; Vejchapipat et al, 2002; Hassoun et al, 2003; Attuwaybi et al, 2003), but also with prevention of hepatic bioenergetic failure (Vejchapipat et al, 2001), preservation of myocardial metabolism (see Chapter 3), reduction in lung neutrophil infiltration (Vinardi et al, 2003), and decreased oxidative stress in plasma, lungs and kidneys (see Chapter 3). However, hypothermia throughout I/R injury can only be applied in elective procedures where mesenteric ischaemia can be predicted in advance (e.g. cardiac surgery using cardiopulmonary bypass, aortic aneurysm repair and intestinal transplantation). In the majority of clinical conditions associated with intestinal I/R, therapy can be commenced only after intestinal ischaemia, thus therapy must be effective when applied at or after the time of reperfusion. Investigation of a possible *rescue* therapy for acute intestinal I/R injury is of clinical relevance, and an experimental study was therefore designed to assess the effects of moderate hypothermia, applied as a rescue therapy at the beginning of reperfusion, in a rat model of intestinal I/R injury.

5.2 MATERIAL AND METHODS

5.2.1 Study design

Young adult male Sprague-Dawley rats weighing between 250 and 300g were used. Animals were kept under standard conditions for food, water, light and temperature, and had access to standard rat chow and water *ad libitum* until the beginning of the experiment.

Study A

Animals were randomly assigned to one of five experimental groups (n=6 per group):

- A) Control Normothermia: sham operation and observation for 360min at normothermia
- B) I/R Normothermia: 60min intestinal ischaemia at normothermia, followed by 300min normothermic reperfusion
- C) Control Hypothermia: sham operation and observation; 60min at normothermia, followed by 300min at hypothermia
- D) I/R Hypothermia: 60min intestinal ischaemia at normothermia, followed by 300min hypothermic reperfusion
- E) I/R Hypothermia with Rewarming: 60min intestinal ischaemia at normothermia, followed by 120min hypothermic reperfusion, and 180min rewarming.

Study B

Animals were randomly assigned to one of four experimental groups (n=8 per group):

- A) Control Normothermia: sham operation and observation for 180min at normothermia
- B) I/R Normothermia: 60min intestinal ischaemia at normothermia, followed by 120min normothermic reperfusion
- C) Control Hypothermia: sham operation and observation; 60min at normothermia, followed by 120min at hypothermia

- D) I/R Hypothermia: 60min intestinal ischaemia at normothermia, followed by 120min hypothermic reperfusion

Rectal temperature was monitored continuously throughout the experiment from induction of anaesthesia. Normothermia (rectal temperature between 36 and 38°C) was maintained by use of a heating blanket and lamp as discussed in Chapter 2. Moderate hypothermia (rectal temperature between 30 and 32°C) was induced at the beginning of reperfusion by cooling the animals with a fan in an environmental temperature of 22 to 23°C; target temperature (32°C) was reached within 20 to 30min (median 26min), and warming applied as necessary to prevent excessive cooling (Figure 5.1). Rewarming was achieved in the I/R Hypothermia with Rewarming group over 60min and animals maintained between 36 and 38°C until the end of the experiment.

5.2.2 Surgical Procedure

Inhalation of isoflurane in an oxygen and nitrous oxide mixture was used for general anaesthesia as discussed in Chapter 2.

A pulse oximeter (NonIn Medical, Minneapolis, MN) was placed on the right foot to monitor peripheral oxygen saturation. A tracheostomy was then performed by inserting a 16G cannula (BD Neoflon, Helsinborg, Sweden) into the trachea through a midline cervical incision. Animals were then ventilated with a small animal ventilator (CWE, Ardmore, PA) and gas flow was adjusted in order to maintain peripheral oxygen saturation >95%. Ventilatory requirements in the different groups, as well as accuracy of the pulse oximeter, were assessed in a preliminary set of experiments by monitoring arterial blood gases (iStat Corporation, Princeton, NJ, USA).

Study A

Once animals were stabilised, the right femoral vessels were exposed with a longitudinal incision. A 24G cannula (BD Neoflon, Helsinborg, Sweden) was inserted into the right femoral artery, and the line was maintained patent by continuous infusion of 0.9% normal saline (10ml/kg/h). Arterial pressure and heart

rate were monitored throughout with a transducer connected to the arterial line. Data were recorded and analysed with a Transonic T206 (Transonic Systems Inc., Ithaca, NY), and mean arterial pressure (MAP) was calculated; results were recorded at 15min intervals.

After insertion of the femoral line, intestinal ischaemia and reperfusion or sham operation were performed as described in Chapter 2.

Survival was then monitored for up to 300min from the onset of reperfusion, and experimental animals were considered dead when systolic pressure was <20mmHg and heart rate was <50bpm; survival time from the beginning of reperfusion was recorded. Rats that were still alive at the end of the experiment were killed by exsanguination under terminal anaesthesia.

Resuscitation with boluses of normal saline through the arterial femoral line was performed at the following time points: immediately after femoral line insertion (3ml/kg); 30 min after the beginning of ischaemia (1.5ml/kg); and 5, 60, 120, 180, and 240 min after the beginning of reperfusion (3ml/kg each).

Study B

Once animals were stabilised, the right femoral vessels were exposed with a longitudinal incision. A 24G cannula (BD Neoflon, Helsinborg, Sweden) was inserted into the right femoral vein, and the line was maintained patent by continuous infusion of 0.9% normal saline (10ml/kg/h).

After insertion of the femoral line, sham operation and superior mesenteric artery occlusion-deocclusion were performed as described in Chapter 2.

Resuscitation with boluses of normal saline through the venous femoral line was performed at the following time points: immediately after femoral line insertion (3ml/kg); 30min after the beginning of ischaemia (1.5ml/kg); and 5, and 60min after the beginning of reperfusion (3ml/kg each).

One min before the end of the experiment, animals were heparinised by infusion of 1,000 IU/kg sodium heparin in 5ml/kg 0.9% saline via the femoral line. Animals were then sacrificed by exsanguination under terminal anaesthesia. A liver sample was freeze-clamped in liquid nitrogen for measurements of phosphoenergetics. The heart was removed and placed in ice-cold medium for total

CPT activity measurement. A blood sample was collected from the thorax, transferred into a heparin tube and centrifuged (3000rpm for 5min at 4°C), and plasma was stored at -70°C until analysis. Small intestine (10-25cm from the ileocaecal valve) and lungs were also removed and stored at -70°C. Terminal ileum (5 to 10cm from the ileocaecal valve) was fixed in 10% formaldehyde for 24 hours and embedded in paraffin.

5.2.3 Tissue extraction and metabolite measurement

Liver phosphoenergetics were quantified by HPLC. Myeloperoxidase activity in ileum and lungs was measured by spectrophotometry. Levels of malondialdehyde in terminal ileum, lungs and heart were determined by HPLC. The concentration of reduced and oxidised glutathione in ileum was measured by HPLC. Plasma concentration of nitrate plus nitrite, reflecting systemic production of nitric oxide, was quantified by HPLC. Total carnitine palmitoyl transferase activity in the heart was measured by radiochemical assay. Assays for phosphoenergetics, MDA, glutathione, nitrate plus nitrite, and total CPT activity were performed as described in Chapter 2.

Haematoxylin and eosin staining was performed on 5µm sections from paraffin embedded samples of terminal ileum as described in Chapter 2. The degree of histological injury was assessed semi-quantitatively as specified in Chapter 2.

5.2.4 Statistical analysis

Survival from the beginning of reperfusion is expressed as median [range], and results compared by Log rank test. Repeated measures ANOVA with Bonferroni post-test was used to compare values of MAP and heart rate with baseline values within the same experimental group. Histological injury is presented as median [range] and compared by Kruskal-Wallis with Dunn's post-test. All other data are expressed as mean \pm SEM, and 1-way ANOVA with Tukey post-test was used for comparisons. Results showing p values <0.05 were considered significant.

5.3 RESULTS

Study A

5.3.1 Survival

All control animals, normothermic and hypothermic, were alive after 300min reperfusion (Figure 5.2). Conversely, intestinal I/R at normothermia induced death of all experimental animals at 97 to 197min (average 148min) from the beginning of reperfusion. However, moderate hypothermia applied at reperfusion, even when followed by rewarming, abolished mortality: all hypothermic I/R rats with or without rewarming, were alive at 300min reperfusion ($p=0.0005$ vs. I/R normothermia).

5.3.2 Haemodynamics

Mean arterial pressure and heart rate remained at baseline level (around 100mmHg and 350bpm respectively) throughout the experiment in normothermic animals undergoing sham operation (Figure 5.3 A, 5.4 A). However, reduction of body temperature in control animals was associated with a significant reduction in heart rate and, to a lesser extent, in MAP, with values reaching a nadir 60min after the beginning of cooling and then remaining around 280bpm and 90mmHg until sacrifice (Figure 5.3 B, 5.4 B).

In rats undergoing normothermic I/R, heart rate increased and MAP decreased progressively from the beginning of reperfusion despite fluid resuscitation (Figure 5.3 C, 5.4 C). Although a high variability was observed between individual animals, at 3 hours from the beginning of reperfusion average heart rate was as high as 450bpm, and MAP as low as 50mmHg. All animals in this group developed progressive severe hypotension and tachycardia, ultimately resulting in cardiovascular failure and death.

Interestingly, haemodynamic changes in hypothermic I/R rats did not differ significantly from those of hypothermic controls, with heart rate decreasing to approximately 270bpm and MAP to 85mmHg over the first hour of reperfusion (Figure 5.3 D, 5.4 D). In the next four hours of reperfusion, only a marginal decrease in MAP was observed, with no rats developing tachycardia or

hypotension. Rewarming of hypothermic I/R animals after 120min of hypothermic reperfusion was followed by a rapid increase in both heart rate and MAP, so that at the end of rewarming haemodynamics were restored to baseline levels in all experimental animals (Figure 5.3 E, 5.4 E). Animals remained haemodynamically stable thereafter, and at sacrifice after 300min reperfusion animals in this group averaged 350bpm and 90mmHg, with little variability within the group.

Study B

5.3.3 Histological injury in ileum

Ileum histology showed normal mucosa or minimal epithelial slough at villous tips in both normothermic and hypothermic controls (Figure 5.5; Figure 5.6 A, C). Intestinal architecture was profoundly damaged following normothermic I/R (Figure 5.5, $p < 0.001$ vs. controls): deep damage to the mucosa with complete disruption of intestinal villi, and congestion of blood vessels in the submucosa were visible in all specimens (Figure 5.6 B), whereas signs of haemorrhage and damage to the lamina propria, and considerable infiltration of leukocytes in the submucosa were present in half of the sections. Hypothermia afforded partial protection to mucosal injury in I/R animals (Figure 5.5, $p = \text{n.s.}$ vs. controls, $p = \text{n.s.}$ vs. I/R Normothermia): damage was limited to the tip or the core of the villi, without involving the crypts or the base of villi (Figure 5.6 D), with only modest hyperaemia and few leukocytes infiltrating the submucosa.

5.3.4 Neutrophil infiltration

Intestinal I/R at normothermia was characterised by a huge increase in the degree of neutrophil infiltration in both ileum (Figure 5.7 A) and lungs (Figure 5.7 B). Moderate hypothermia following intestinal I/R effectively reduced neutrophil infiltration in both organs as compared to I/R at normothermia. This effect was more marked in the ileum, where MPO activity in hypothermic I/R rats was as low as in controls.

Assessment of neutrophil infiltration by MPO activity in the intestine was supported by microscopic evaluation of specimens from terminal ileum, with rats

showing the highest values of MPO in the intestine also presenting a considerable number of leukocytes visible at haematoxylin and eosin staining.

5.3.5 Lipid peroxidation

Lipid peroxidation developed as a consequence of intestinal I/R in plasma (Figure 5.8 A), ileum (Figure 5.8 B) and lungs (Figure 5.8 C) of normothermic rats compared to controls. Moderate hypothermia counteracted the increase of lipid peroxidation in the ileum, but systemic and pulmonary levels of MDA were similar in I/R rats at either normothermia or hypothermia. The degree of lipid peroxidation in the heart was similar in all experimental groups and unaffected by either mesenteric ischaemia or change in body temperature (Figure 5.8 D).

5.3.6 Glutathione content

Intestinal I/R at normothermia was associated with a substantial decrease of antioxidant potential in the ileum, as suggested by the drastic decrease in the concentrations of reduced (Figure 5.9 A), oxidised (Figure 5.9 B) and total glutathione (Figure 5.9 C) with respect to controls, suggesting severe antioxidant depletion. Rescue hypothermia conferred only limited protection, preventing the decrease in oxidised and, to some extent, reduced glutathione. However, only a non-significant trend to improvement was observed in levels of total glutathione of hypothermic I/R rats compared to I/R at normothermia (Figure 5.9 C). No changes in glutathione redox state in the ileum were seen between the four experimental groups (Figure 5.9 D).

5.3.7 Systemic nitric oxide production

Systemic nitric oxide production was increased following intestinal I/R at normothermia compared to normothermic controls (Figure 5.10), but this effect was not observed in hypothermic I/R rats.

A highly significant positive correlation ($r^2=0.2085$; $p=0.0086$) was observed between systemic NO production and plasma levels of MDA between all samples (Figure 5.11).

5.3.8 *TNF- α production*

Plasma levels of TNF- α were undetectable in all normothermic and all but one hypothermic controls (Figure 5.12), reflecting low systemic release following sham operation. Reperfusion of the intestine induced a marked increase in TNF- α concentration in plasma, suggesting an intense inflammatory response, with similar levels in both normothermic and hypothermic rats.

5.3.9 *Liver phosphoenergetics*

Intestinal I/R at normothermia caused a significant decrease in hepatic ATP (Figure 5.13 A), with a parallel rise in AMP (Figure 5.13 C) and inorganic phosphate (Figure 5.13 E). Depletion in high energy phosphates was confirmed by the drop in liver energy charge (Figure 5.13 F), whereas total adenine nucleotides (ATP+ADP+AMP) were unaffected by normothermic intestinal I/R (Figure 5.13 D). Moderate hypothermia completely prevented the development of hepatic energy failure: there was no alteration in ATP, ADP, AMP, energy charge or inorganic phosphate compared to controls.

5.3.10 *Carnitine palmitoyl transferase activity in the heart*

Intestinal ischaemia and reperfusion at normothermia significantly inhibited heart total CPT activity compared to normothermic control animals (Figure 5.14). Even though initiation of hypothermia was delayed until the beginning of the reperfusion phase, and not completed until about 30min of reperfusion, total CPT activity in I/R animals at hypothermia was significantly higher than in I/R animals at normothermia, and comparable to controls at both normothermia and hypothermia.

5.4 DISCUSSION

Moderate hypothermia has been shown to provide robust protection to distant organs, as shown in Chapter 3, and improve survival (Vejchapipat et al, 2001; Kalia et al, 2002b) from intestinal I/R, but only when applied throughout both ischaemia and reperfusion. In the present study, I have shown therapeutic

hypothermia to be effective when applied as a rescue therapy after the onset of intestinal reperfusion. This may make the clinical use of moderate hypothermia applicable to conditions in which patients can only be treated after the reperfusion phase, rather than before the onset of ischaemia. In my study, although I started to cool animals at the time of reperfusion, target temperature was achieved only after 20-30min, suggesting that there may be a “window of opportunity” following initiation of reperfusion during which intervention can be effective. The results indicate that rescue moderate hypothermia applied after reperfusion following normothermic ischaemia can dramatically improve survival and maintain adequate cardiovascular function, preventing development of severe tachycardia and hypotension. This suggests that a considerable part of damage is exerted during the reperfusion phase rather than during ischaemia, as in other ischaemia and reperfusion models. Importantly, these beneficial effects remained even when animals were slowly rewarmed, and there did not appear to be any deleterious rebound effect caused by rewarming, such as peripheral vasodilatation with lowered blood pressure (Thoresen et al, 2000).

Although in previous experiments of hypothermia I have used spontaneously breathing animals, as have other groups (Kalia et al, 2002b; Hassoun et al, 2002), in preliminary experiments of hypothermia applied at reperfusion, animals that were not ventilated experienced fatal respiratory arrest. This could be due to a decreased spontaneous respiratory activity when body temperature falls below 32°C, resulting both in severe hypercapnia with respiratory acidosis, and in profound hypoxia (Connolly et al, 2000). Clearly, in clinical application of therapeutic hypothermia, all patients would be ventilated and closely monitored during the cooling phase.

Intestinal I/R produces a systemic inflammatory response with multiple organ failure. The beneficial effects observed in this study could have resulted from direct protection of distant organs and/or from the reduction of systemic inflammatory response secondary to effects on reperfused intestine. Hypothermia partially prevented gut histological injury and completely prevented neutrophil infiltration, consistent with attenuation of leukocyte endothelial adherence and chemotactic activity by hypothermia (Kimura et al, 2002; Kalia et al, 2002b).

Hypothermia also protected against oxidative stress in the ileum, which could be due to reduced recruitment of neutrophils, or impairment in free radical production by activated leukocytes (Frohlich et al, 2004). When hypothermia was applied throughout I/R, intestinal glutathione was maintained by hypothermia (see Chapter 3), whereas in the current study, there was only a minimal effect of hypothermia on gut glutathione concentration, suggesting that the decrease in glutathione occurs during ischaemia or early reperfusion, and that glutathione is not fundamental in the mechanism of protection. Prevention of oxidative stress following I/R may depend more on decreased free radical production than on increased antioxidant potential.

Hepatic failure is a component of multiple organ failure following intestinal reperfusion injury, and it has been shown that development of liver energy failure immediately precedes death of animals undergoing intestinal I/R (Exon et al, 1975), suggesting coupling of hepatic energy status to multiple organ failure. Maintenance of ATP production is key to an organ with a high metabolic activity such as the liver (Jaeschke et al, 2003), and decrease of hepatic ATP levels is an early indicator of liver injury and a predictor of outcome in acute liver failure in rats (Corbin et al, 2003). Rescue hypothermia completely prevented development of hepatic energy failure observed after two hours of normothermic reperfusion.. This protective effect could be due to improvement in hepatic haemodynamics: during intestinal reperfusion, total hepatic blood inflow is reduced by approximately 66% mostly due to a reduction in arterial blood supply, which would contribute to hepatic dysfunction (Turnage et al, 1996). Alternative mechanisms possibly responsible for hepatic protection include inhibition of pro-apoptotic and pro-inflammatory transcriptional factors, which are modulated by hypothermia when applied throughout intestinal ischaemia and reperfusion (Parkinson et al, 2004).

Propagation of a systemic inflammatory response from gut and liver to other organs could be mediated via cytokines or other factors (Oberholzer et al, 2000). In these experiments, hypothermia did not reduce systemic TNF- α levels, although I did not measure other cytokines. Activation of nitric oxide synthase and/or endothelium are crucial aspects of a systemic inflammatory response, and

increased nitric oxide production is associated with organ failure (Doughty et al, 1998; Brealey et al, 2004) and poor outcome (Brealey et al, 2002; Langouche et al, 2005). Systemic nitric oxide production was greatly increased by intestinal I/R, and this increase was prevented by hypothermic reperfusion. Although the role of NO during intestinal reperfusion is multi-faceted and includes beneficial effects such as reduction of leukocyte adhesion and vasodilatation (Kalia et al, 2001; Horie et al, 1998), overproduction of NO is deleterious and leading to formation of peroxynitrite (Cuzzocrea et al, 2000).

Compromise of respiratory function due to capillary leak and pulmonary oedema is frequent following intestinal I/R (Harward et al, 1993). Lung neutrophil infiltration is a major determinant of microvascular leak following intestinal reperfusion (Kadesky et al, 1995; Koike et al, 1995; Terada et al, 1992) and acute respiratory distress syndrome (Pugin et al, 1999; Ishizaka et al, 2001; Steinberg et al, 1994), contributing to capillary leak by producing reactive oxygen species, proteolytic enzymes, and a range of mediators that enhance the inflammatory response (Ley, 2004; Hansen, 1995). Rescue hypothermia dramatically decreased pulmonary neutrophil infiltration, potentially preventing development of respiratory failure. Although free radical production by neutrophils contributes to the development of oxidative stress, reduction in pulmonary neutrophil infiltration by rescue hypothermia was not associated with decreased lipid peroxidation, suggesting that other sources of reactive species are unaffected by hypothermia.

Primary respiratory failure with impaired gas exchange could have contributed to progressive deterioration of cardiovascular function despite adequate ventilatory support. However, other mechanisms could also be responsible for the observed development of cardiovascular derangement. Primary dysfunction of both myocardial contraction and relaxation has been reported following intestinal I/R, and this can be prevented by free-radical scavengers and inhibitors of neutrophil adherence (Horton et al, 1991; Horton et al, 1993a; Horton et al, 1993b). Although in my previous experiments cardiac energy failure does not occur within 2 hours of reperfusion (see Chapter 3), I have previously shown that CPT activity, crucial for the control of mitochondrial utilisation of fatty acids (the major fuel source for the myocardium) is decreased following intestinal I/R (see

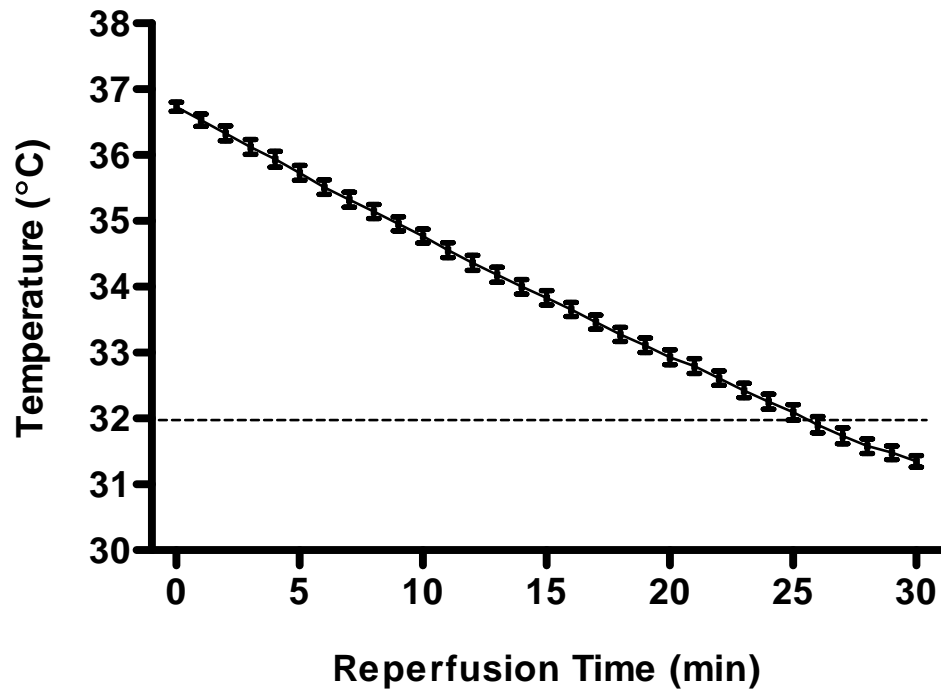
Chapter 4) and sepsis (Fukumoto et al, 2002a) due to peroxynitrite (Fukumoto et al, 2004). Total CPT activity was decreased by I/R at normothermia, and this effect was prevented by rescue hypothermia, which would allow a normal rate of fatty acid oxidation and could help to prevent later cardiac energy failure.

In the current experiment, I measured total CPT activity (i.e. CPT I plus CPT II) as hearts were frozen before analysis, making it impossible to evaluate CPT I and CPT II activity separately. In previous experiments, CPT I activity was measured separately from CPT II, and a decrease in CPT I activity with unaltered CPT II activity was found (see Chapter 4; Fukumoto et al, 2004; Fukumoto et al, 2002a). It is therefore likely that changes in total CPT activity in the current study reflect changes in CPT I rather than CPT II activity.

5.5 CONCLUSIONS

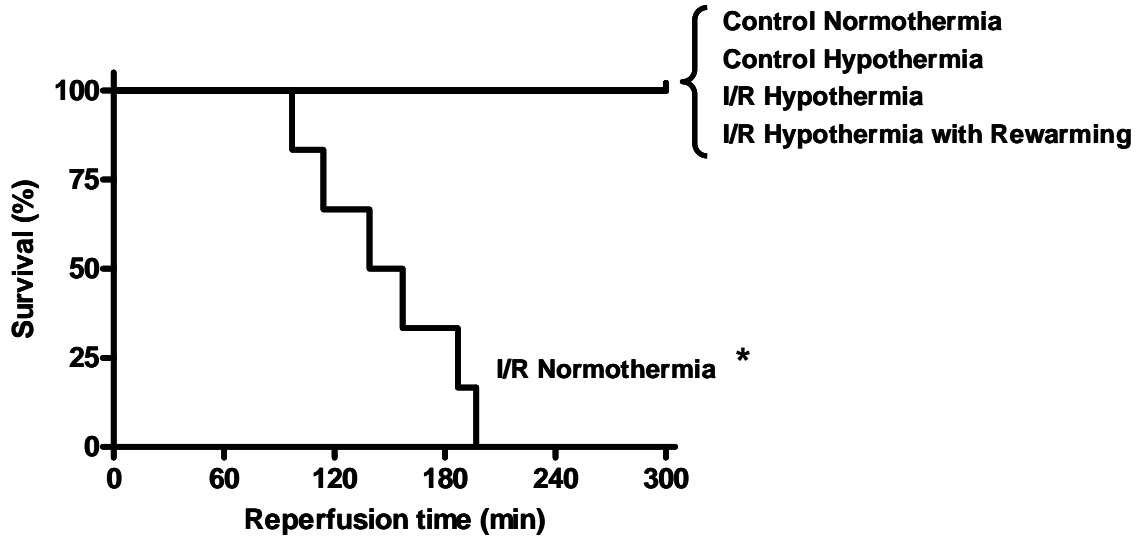
This study demonstrates that whole-body moderate hypothermia applied as a rescue therapy at the beginning of reperfusion improves outcome and prevents cardiovascular failure in a rat model of acute intestinal ischaemia and reperfusion injury. Importantly, hypothermia did not appear to just delay cardiovascular failure, as rewarmed rats also survived to the end of experiment. Investigation of the early reperfusion phase showed that protection provided by rescue hypothermia could result from the modulation of several different pathways involved in determining not only injury to the reperfused ileum, but also multiple organ dysfunction syndrome. Rescue hypothermia proved a robust method to prevent development of energy failure in the liver, systemic overproduction of nitric oxide, infiltration of inflammatory cells in the lungs and impaired cardiac fatty acid utilisation. These observations suggest that moderate hypothermia could potentially be beneficial as a treatment for clinical conditions associated with acute intestinal I/R injury, even when initiated after the ischaemic insult has been established.

Figure 5.1. Core body temperature during the cooling phase.



Core body temperature of rats in the hypothermia groups (n=18) during the cooling phase. Cooling was started at the onset of reperfusion, and target temperature (dotted line) was reached within 30min in all animals. Data are reported as mean \pm SEM.

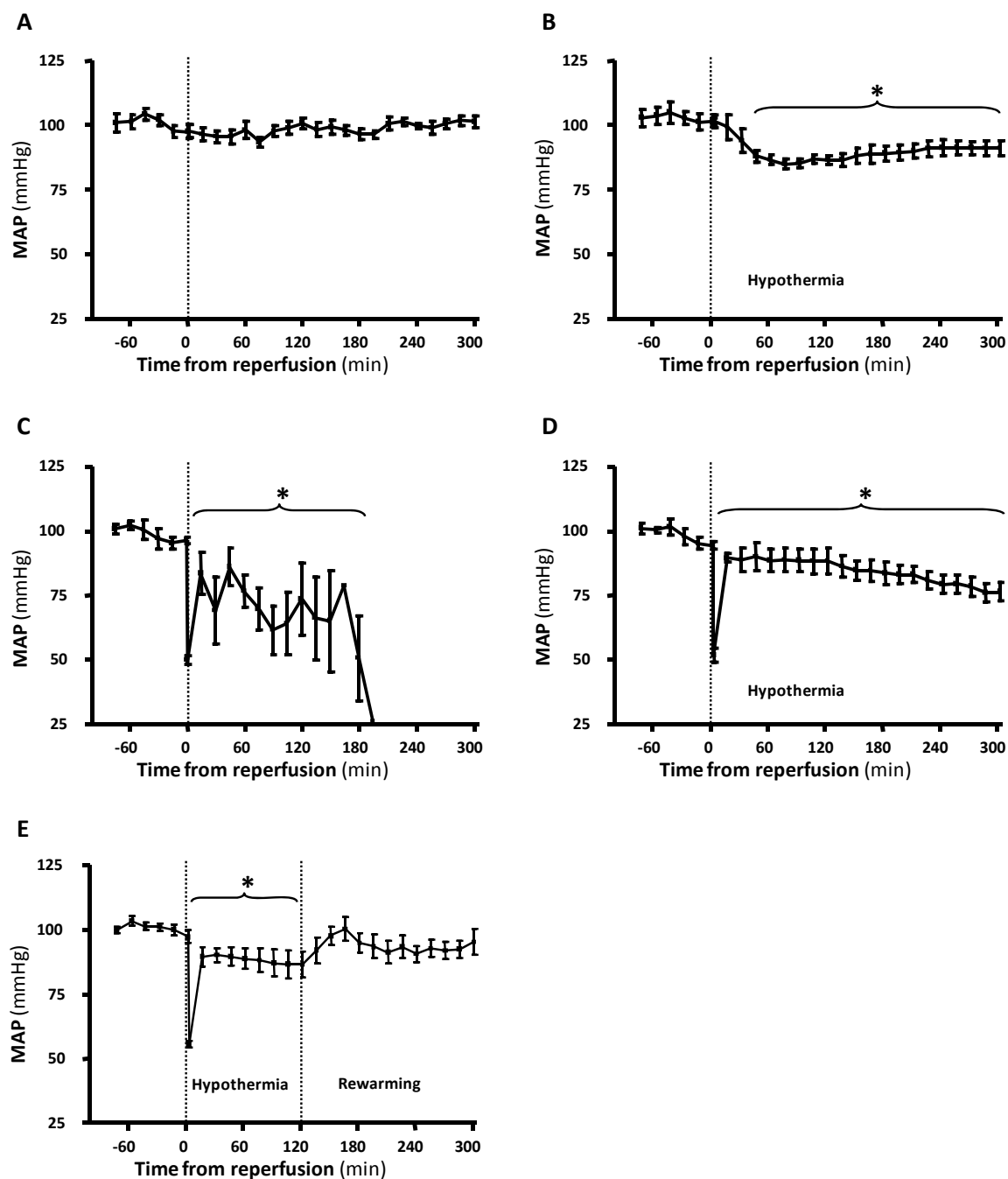
Figure 5.2. Survival of experimental animals.



Animals (n=6 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia) with or without rewarming. Survival time is calculated from the beginning of reperfusion; results were compared by Log rank test.

* I/R Normothermia: $p=0.0005$ vs. Control Normothermia; $p=0.0005$ vs. Control Hypothermia; $p=0.0005$ vs. I/R Hypothermia; $p=0.0005$ vs. I/R Hypothermia with Rewarming.

Figure 5.3. Mean arterial pressure.

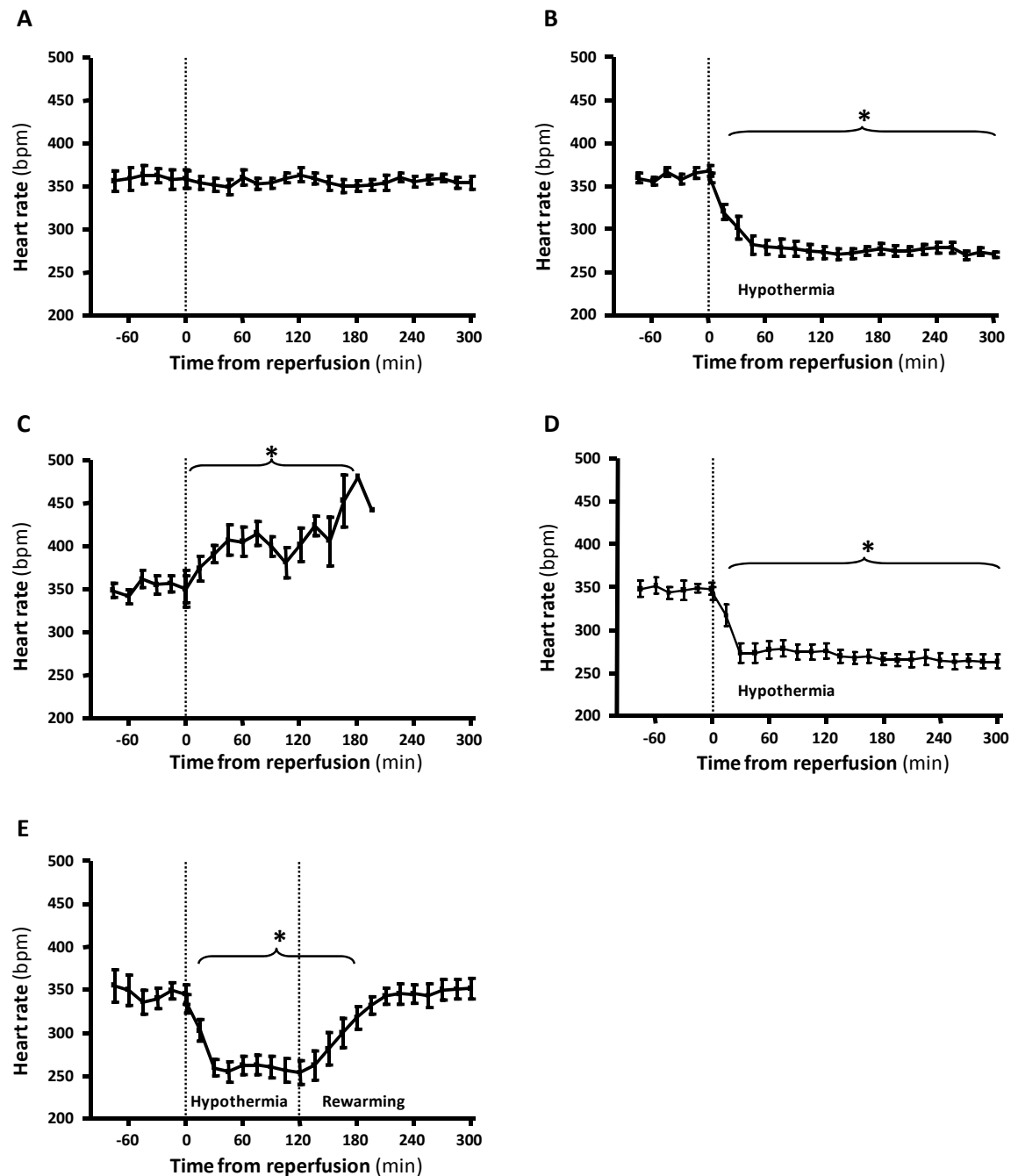


Mean arterial pressure (MAP) was measured invasively in the right femoral artery. Animals (n=6 per group) underwent: sham operation at normothermia (Control Normothermia) [A]; sham operation at hypothermia (Control Hypothermia) [B]; intestinal ischaemia and reperfusion at normothermia (I/R

Normothermia) [C]; intestinal ischaemia and reperfusion at moderate hypothermia (I/R Hypothermia) [D]; or intestinal ischaemia and reperfusion at moderate hypothermia with rewarming (I/R Hypothermia with Rewarming) [E]. MAP is expressed as mmHg. Results are reported as mean \pm SEM and were compared by repeated measurements ANOVA with Bonferroni post hoc test.

* $P < 0.05$ vs. baseline.

Figure 5.4. Heart rate.

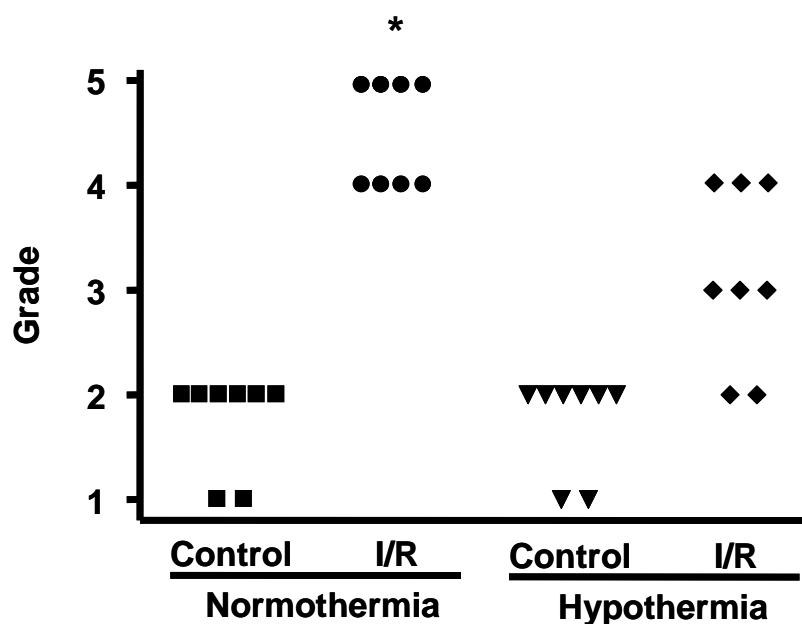


Heart rate was measured invasively in the right femoral artery. Animals (n=6 per group) underwent: sham operation at normothermia (Control Normothermia) [A]; sham operation at hypothermia (Control Hypothermia) [B]; intestinal ischaemia and reperfusion at normothermia (I/R Normothermia) [C]; intestinal

ischaemia and reperfusion at moderate hypothermia (I/R Hypothermia) [D]; or intestinal ischaemia and reperfusion at moderate hypothermia with rewarming (I/R Hypothermia with Rewarming) [E]. Heart rate is expressed as beats per minute (bpm). Results are reported as mean \pm SEM and were compared by repeated measurements ANOVA with Bonferroni post hoc test.

* $P < 0.05$ vs. baseline.

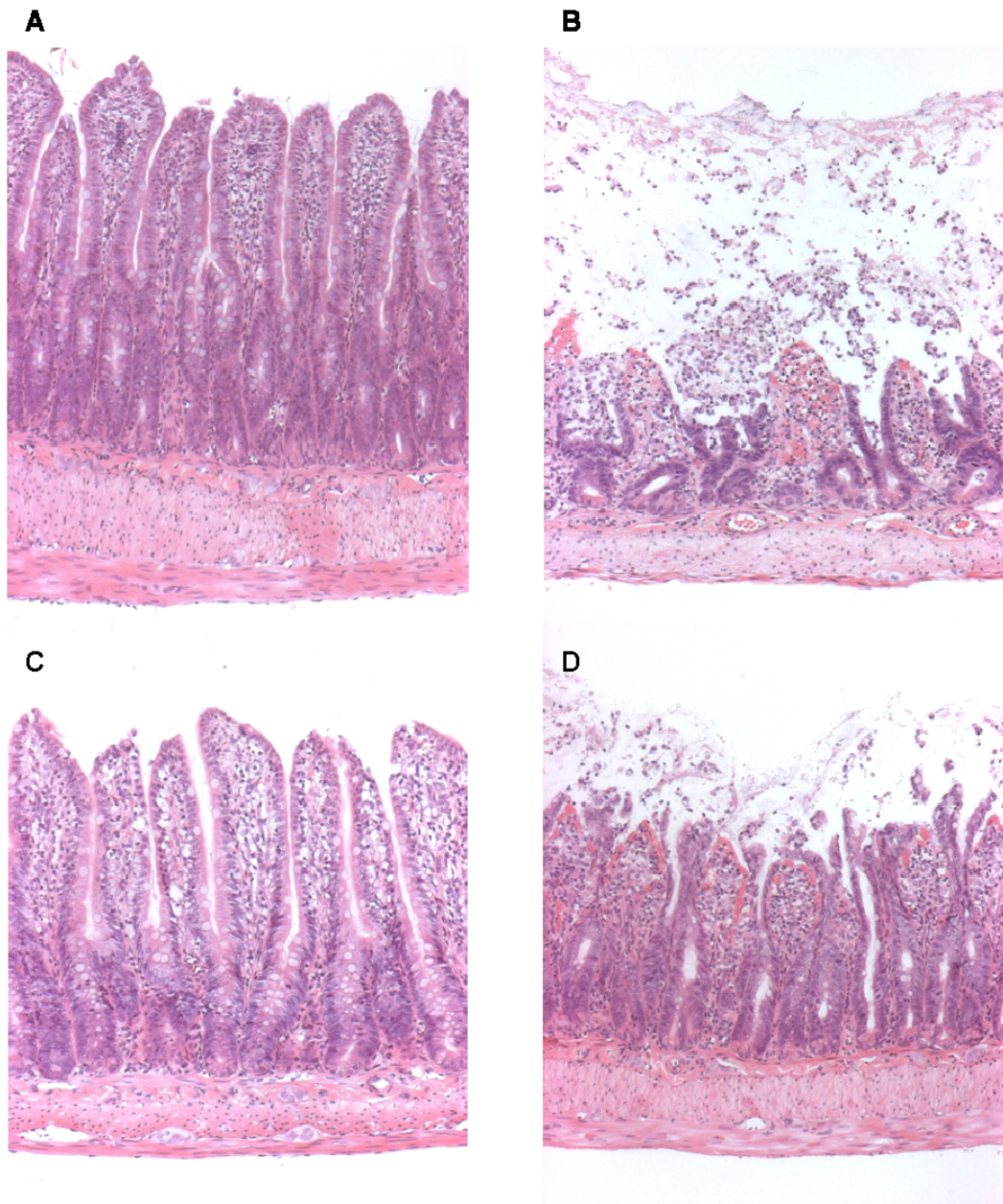
Figure 5.5. Histological injury in the ileum.



Grade of histological injury in the ileum (5=maximum, 1=minimum). Animals (n=8 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia) with or without rewarming. Results were compared using Kruskal-Wallis with Dunn's multiple comparison test.

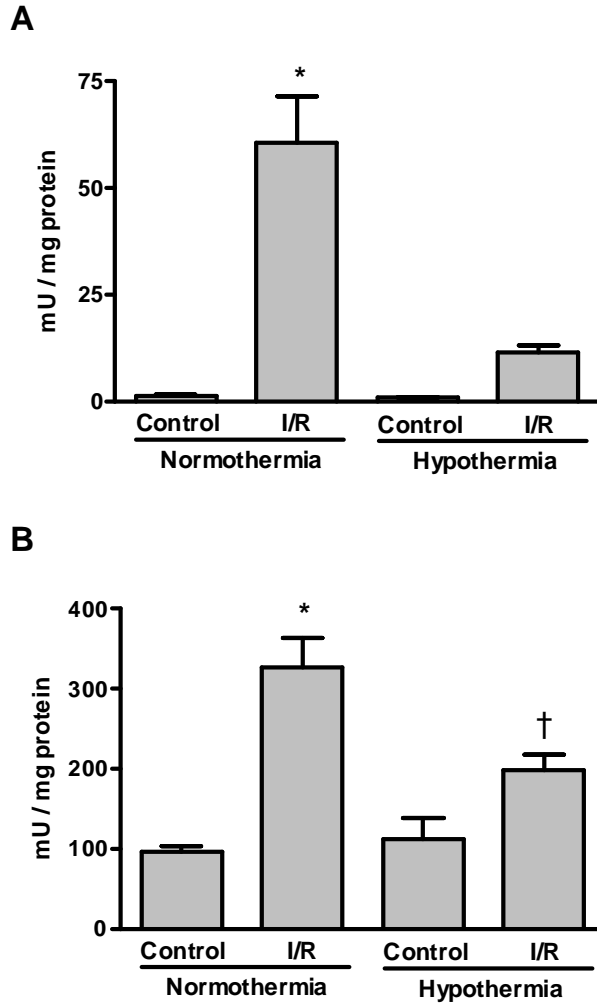
* I/R Normothermia: $p < 0.01$ vs. Control Normothermia, $p < 0.01$ vs. Control Hypothermia

Figure 5.6. Histological appearance of terminal ileum.



Specimens of terminal ileum were stained with haematoxylin and eosin and are from: Control Normothermia [A]; I/R Normothermia [B]; Control Hypothermia [C]; I/R Hypothermia [D]. Magnification is x100.

Figure 5.7. Neutrophil infiltration in ileum and lungs.



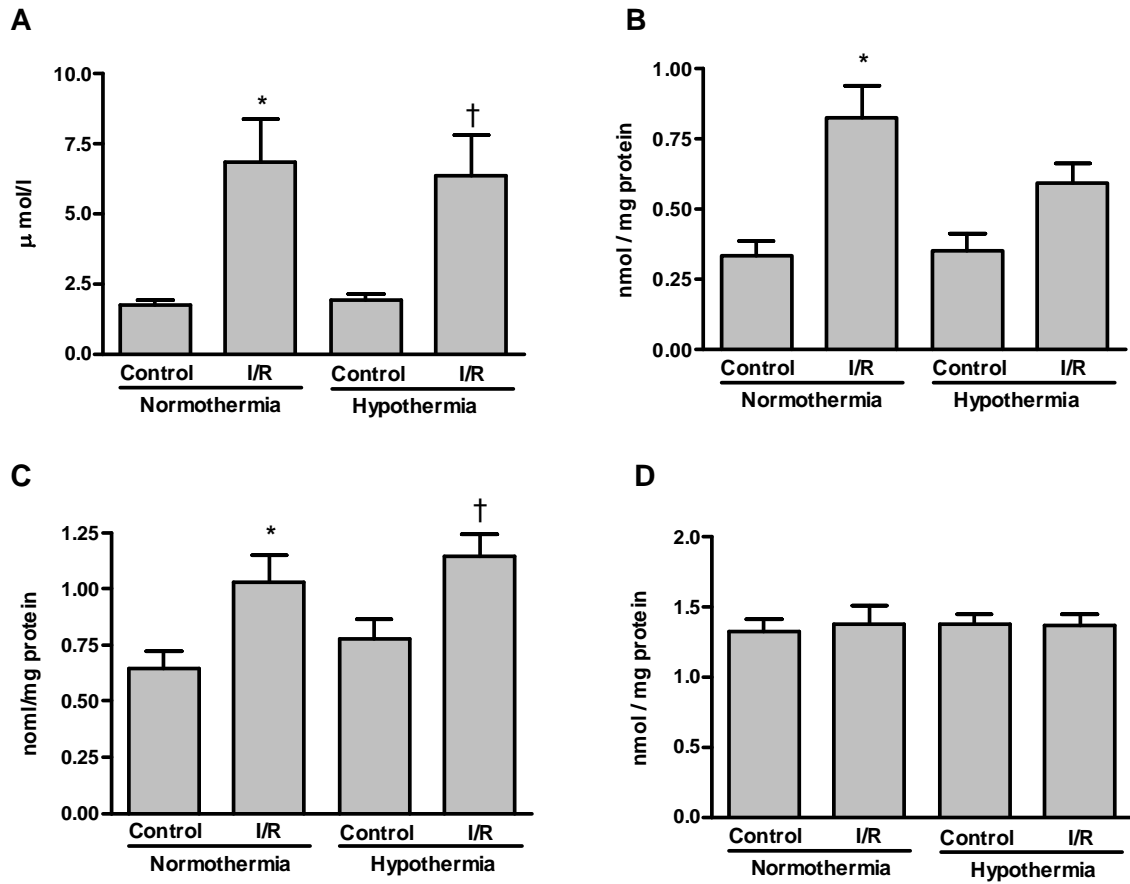
Neutrophil infiltration, assessed as myeloperoxidase activity (MPO) in ileum [A], and lungs [B]. Animals (n=8 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Results are reported as mean \pm SEM; one-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia; $p < 0.001$ vs. I/R Hypothermia.

[B]: * I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia; $p = 0.004$ vs. I/R Hypothermia.

† I/R Hypothermia: $p = 0.024$ vs. Control Normothermia.

Figure 5.8. Malondialdehyde concentration in plasma, ileum, lungs and heart.



Concentration of malondialdehyde (MDA) in plasma [A], ileum [B], lungs [C], and heart [D]. Animals (n=8 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentration of MDA is expressed as $\mu\text{mol/l}$ for plasma, and as nmol/mg protein for ileum, lungs and heart. Results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Normothermia: $p=0.011$ vs. Control Normothermia; $p=0.015$ vs. Control Hypothermia.

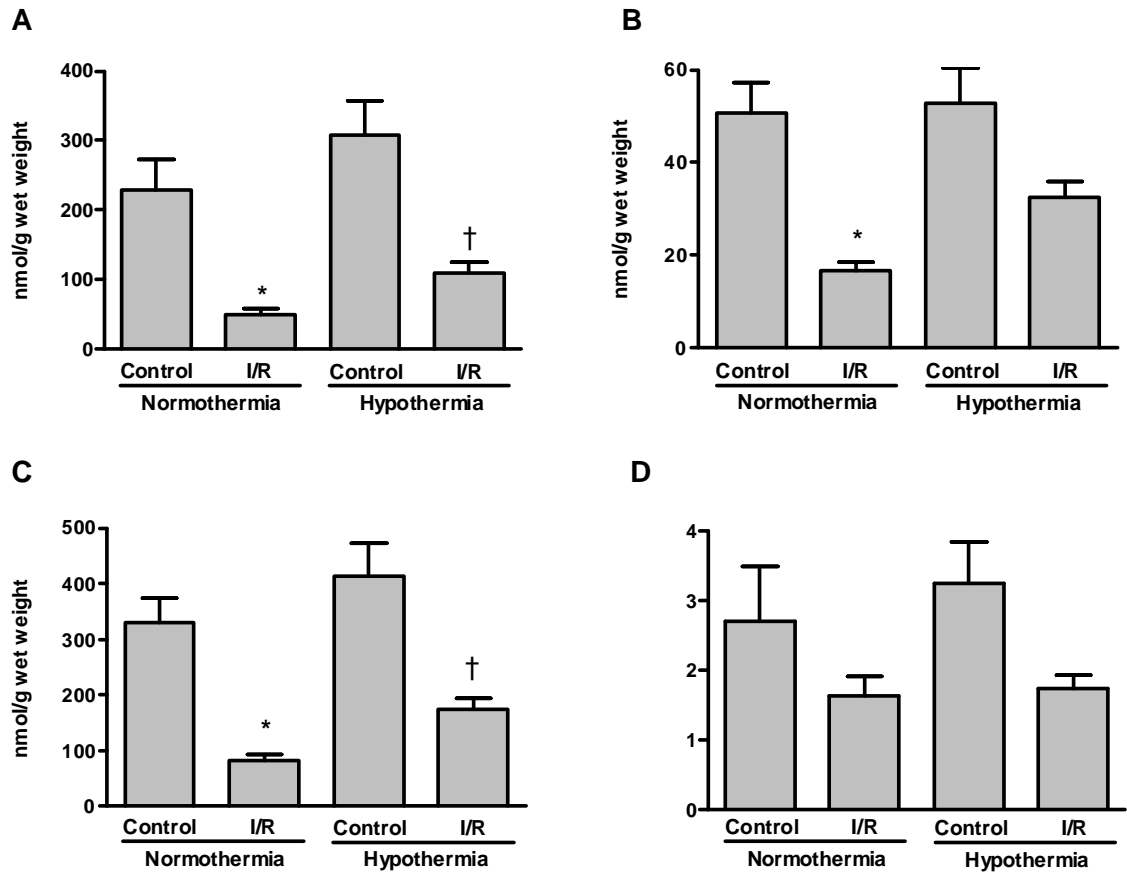
† I/R Hypothermia: $p=0.023$ vs. Control Normothermia; $p=0.031$ vs. Control Hypothermia.

[B]: * I/R Normothermia: $p=0.001$ vs. Control Normothermia; $p=0.001$ vs. Control Hypothermia.

[C]: * I/R Normothermia: $p=0.041$ vs. Control Normothermia

† I/R Hypothermia: $p=0.006$ vs. Control Normothermia.

Figure 5.9. Glutathione concentration in ileum.



Concentration of reduced (GSH) [A], oxidised (GSSG) [B], and total (calculated as $GSH + 2 \times GSSG$) [C] glutathione, and glutathione redox state (calculated as $GSH/2 \times GSSG$) [D] in ileum. Animals (n=8 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentrations of GSH, GSSG and total glutathione are expressed as nmol/g wet weight. Results are reported as mean \pm SEM; one-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Normothermia: $p=0.004$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia.

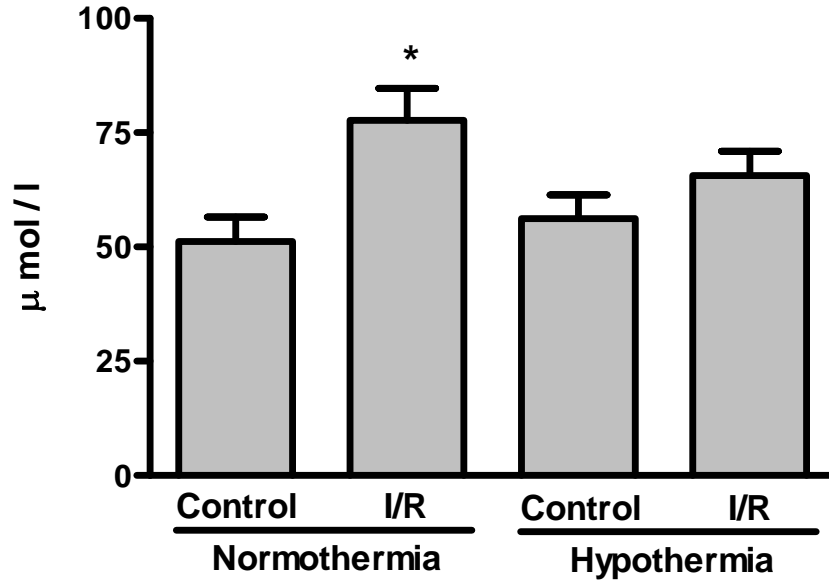
† I/R Hypothermia: $p=0.001$ vs. Control Hypothermia.

[B]: * I/R Normothermia: $p=0.001$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia.

[C]: * I/R Normothermia: $p<0.001$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia.

† I/R Hypothermia: $p=0.034$ vs. Control Normothermia; $p=0.001$ vs. Control Hypothermia.

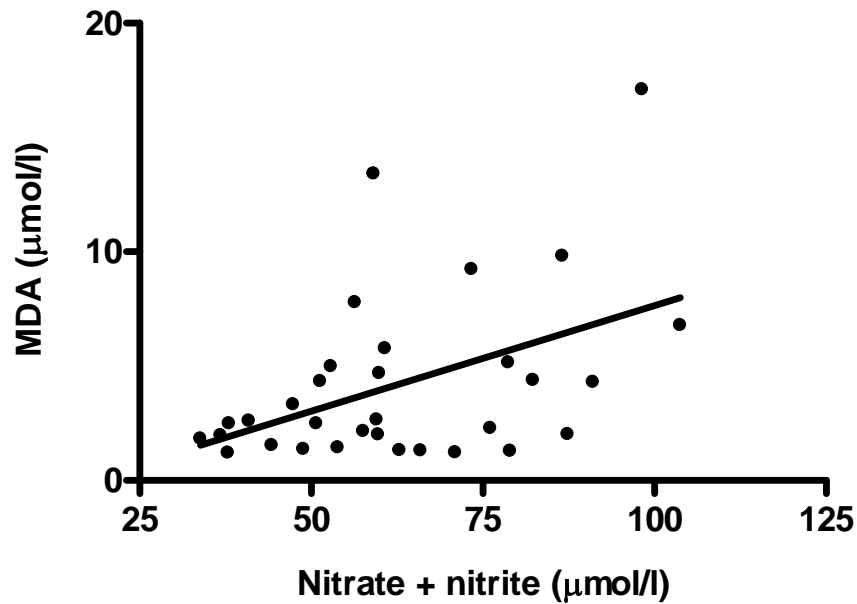
Figure 5.10. Concentration of nitrate plus nitrite in plasma.



Systemic production of nitric oxide measured as concentration of nitrate plus nitrite in plasma. Animals (n=8 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Data are expressed as $\mu\text{mol/l}$ and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

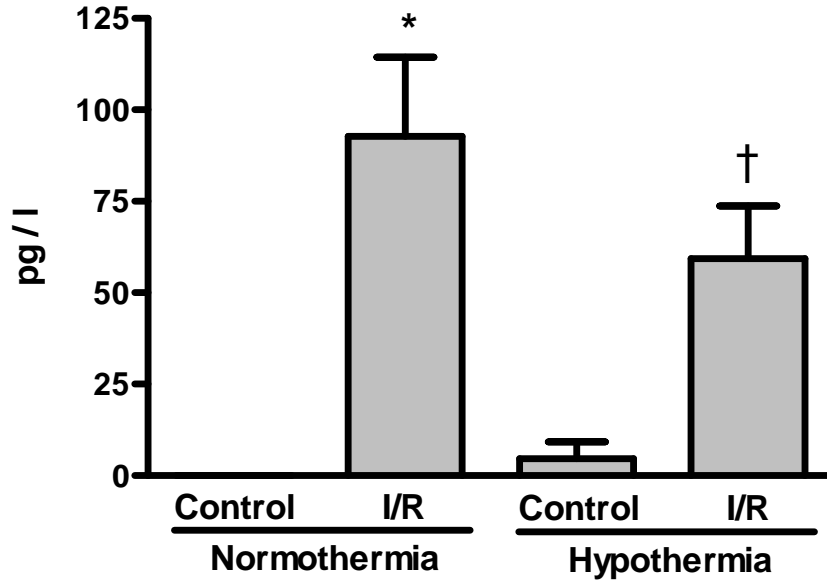
* I/R Normothermia: $p=0.014$ vs. Control Normothermia.

Figure 5.11. Correlation between nitrate plus nitrite and malondialdehyde concentration in plasma.



Correlation between nitrate plus nitrite (expressed as $\mu\text{mol/l}$) and malondialdehyde (MDA, expressed as $\mu\text{mol/l}$) in plasma (linear regression analysis indicated a correlation with $r^2=0.2085$; $p=0.0086$).

Figure 5.12. Concentration of TNF- α in plasma.

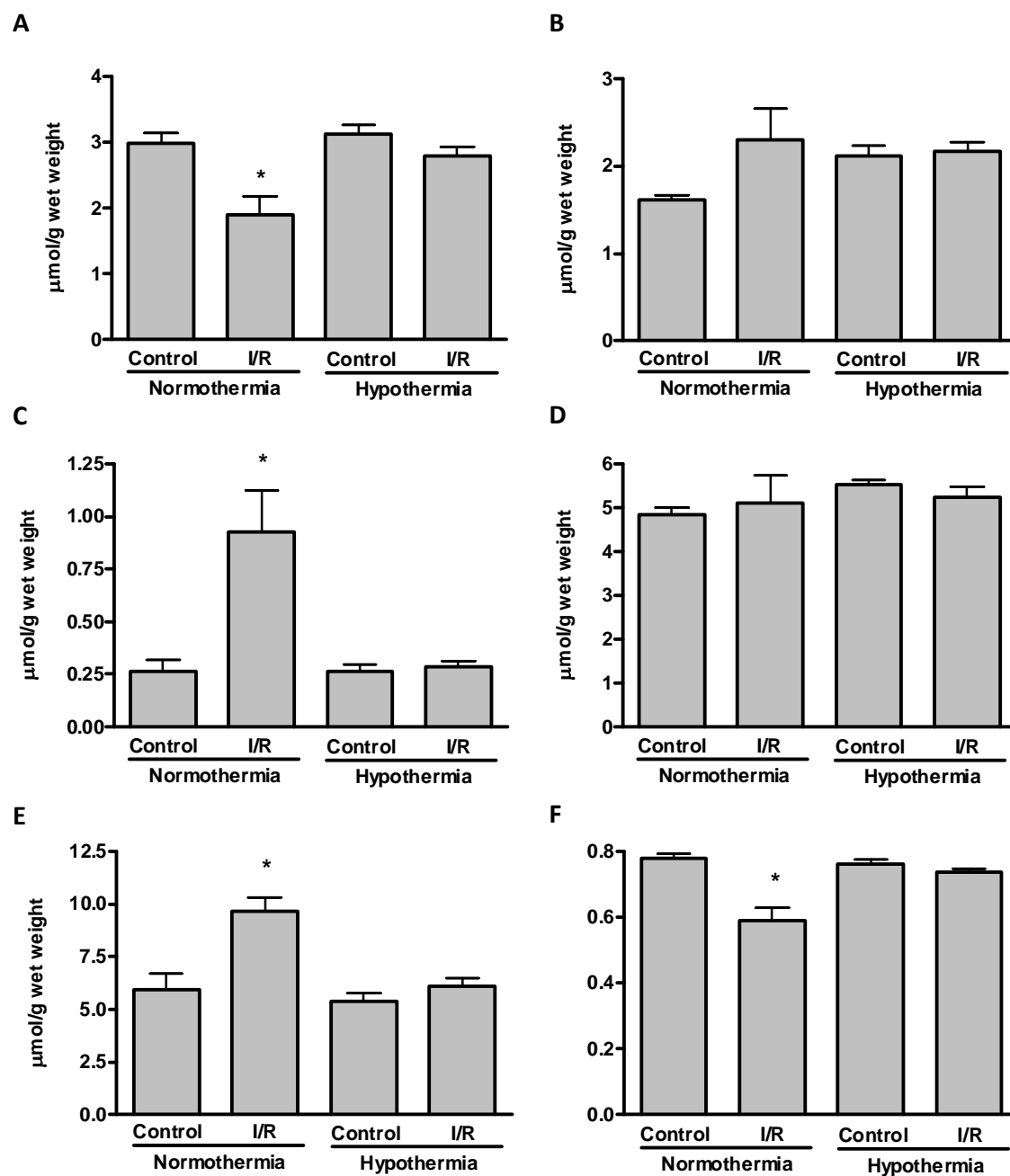


Concentration of TNF- α in plasma. Animals (n=8 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Data are expressed as pg/l and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

* I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia.

† I/R Hypothermia: $p = 0.018$ vs. Control Normothermia; $p = 0.032$ vs. Control Hypothermia.

Figure 5.13. Concentration of phosphoenergetics and energy charge in liver.



Concentration of ATP [A], ADP [B], AMP [C], total adenine nucleotides (calculated as ATP+ADP+AMP) [D], and inorganic phosphate [E], and energy charge (calculated as $[(\text{ATP} + \frac{1}{2}\text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})]$) [F] in liver. Animals (n=8 per group)

underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentrations of ATP, ADP, AMP, total adenine nucleotides, and inorganic phosphate are expressed as $\mu\text{mol/g}$ wet weight and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

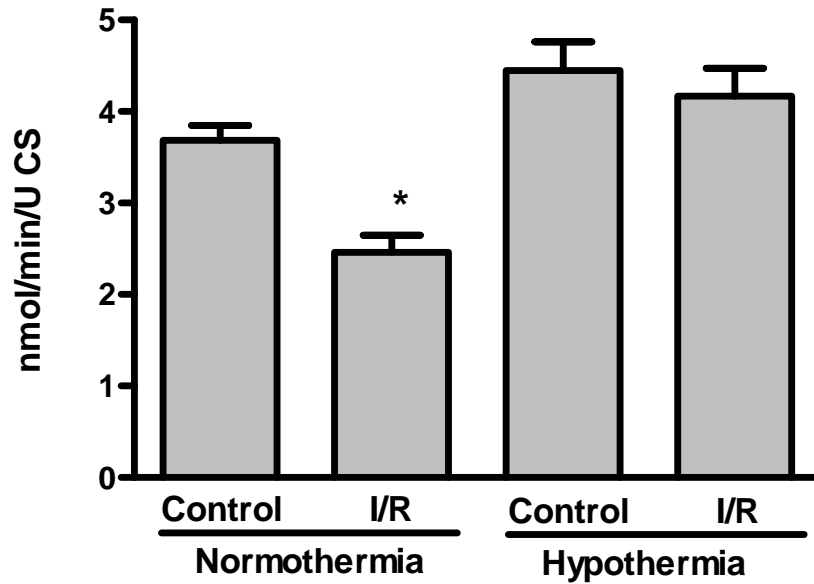
[A]: * I/R Normothermia: $p=0.002$ vs. Control Normothermia; $p=0.001$ vs. Control Hypothermia; $p=0.013$ vs. I/R Hypothermia.

[C]: * I/R Normothermia: $p<0.001$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia; $p<0.001$ vs. I/R Hypothermia.

[E]: * I/R Normothermia: $p<0.001$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia; $p<0.001$ vs. I/R Hypothermia.

[F]: * I/R Normothermia: $p<0.001$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia; $p<0.001$ vs. I/R Hypothermia.

Figure 5.14. Total carnitine palmitoyl transferase in the heart.



Total carnitine palmitoyl transferase (CPT) activity in the heart. Animals underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Data are expressed as mmol/min/U citrate synthase (CS) and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

* I/R Normothermia: $p=0.009$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia; $p<0.001$ vs. I/R Hypothermia.

CHAPTER 6

MODERATE HYPOTHERMIA AS A RESCUE TREATMENT FOR INFANT INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

CHAPTER 6

MODERATE HYPOTHERMIA AS A RESCUE TREATMENT FOR INFANT INTESTINAL ISCHAEMIA AND REPERFUSION INJURY

6.1 BACKGROUND

Previous reports have shown that moderate hypothermia improves survival in adult animals when applied during both the ischaemic and the reperfusion phase (see Chapter 3; Vejchapipat et al, 2001; Kalia et al, 2002b). Interestingly, this hypothermic protection appears to be mediated by preventing MODS through reduction in lung neutrophil infiltration (Vinardi et al, 2003), prevention of liver bioenergetic failure (Vejchapipat et al, 2001), preservation of myocardial energy metabolism (see Chapter 3) as well as decreased oxidative stress in plasma, lungs and kidneys (see Chapter 3). However, in the clinical scenario of neonatal and paediatric conditions associated with intestinal I/R, therapy can usually only be applied after the ischaemic insult has occurred, and any potential treatment should therefore prove to be effective when initiated as a rescue during the reperfusion phase. In Chapter 5, I have shown that the beneficial effects of hypothermia are preserved even when applied as a rescue at reperfusion in an adult animal model. Although little is known regarding gut I/R injury in developing animals, it has been highlighted that the response to a reduced mesenteric flow is substantially different in neonates or infants compared with adults (Luo et al, 2004; Nankervis et al, 2000; Crissinger et al, 1989), suggesting that the beneficial effects of hypothermia observed in adults may not apply to developing animals.

Aim of the current study was therefore to investigate the effects of moderate hypothermia applied as a rescue therapy in an infant model of mesenteric ischaemia and reperfusion.

6.2 MATERIAL AND METHODS

6.2.1 Study design

Suckling mixed-sex Sprague-Dawley rats of 11-13 days of age weighing between 18 and 30g were used. The suckling rats were kept with their dams and allowed to suckle freely until anaesthetised. The family groups were kept under standard conditions for food, light and temperature.

Study A

Animals were randomly assigned to one of four experimental groups (n=8 per group):

- A) Control Normothermia: sham operation and observation for 285min at normothermia
- B) I/R Normothermia: 45min intestinal ischaemia at normothermia, followed by 240min normothermic reperfusion
- C) I/R Hypothermia: 45min intestinal ischaemia at normothermia, followed by 240min hypothermic reperfusion
- D) I/R Hypothermia with Rewarming: 45min intestinal ischaemia at normothermia, followed by 120min hypothermic reperfusion, and 120min rewarming

Study B

Animals were randomly assigned to one of four experimental groups (n=10 per group):

- A) Control Normothermia: sham operation and observation for 135min at normothermia
- B) I/R Normothermia: 45min intestinal ischaemia at normothermia, followed by 90min normothermic reperfusion
- C) Control Hypothermia: sham operation and observation; 45min at normothermia, followed by 90min at hypothermia
- D) I/R Hypothermia: 45min intestinal ischaemia at normothermia, followed by 90min hypothermic reperfusion

Rectal temperature was monitored continuously throughout the experiment from induction of anaesthesia. Normothermia [rectal temperature between 34 and 36°C, which represents the physiological range for suckling rats of this age (Bertin et al, 1993)] was maintained by use of a heating blanket and lamp as described in Chapter 2. Moderate hypothermia (rectal temperature between 30 and 32°C) was induced at the beginning of reperfusion by cooling the animals with a fan in an environmental temperature of 22 to 23°C; target temperature (32°C) was reached within 12min (median 7min, Figure 6.1), and warming applied as necessary to prevent excessive cooling. Rewarming was achieved in the I/R Hypothermia with Rewarming group over 30min and animals maintained between 34 and 36°C until the end of the experiment.

6.2.2 Surgical Procedure

General anaesthesia was induced and maintained by inhalation of isoflurane in an oxygen and nitrous oxide mixture in spontaneously breathing animals as described in Chapter 2.

Mesenteric ischaemia and reperfusion and sham operation were performed under microscopic view as discussed in Chapter 2.

Immediately after closure of the abdomen, the right femoral vessels were exposed through a longitudinal incision, a 0.16mm polyimide catheter (Cole-Parmer, Vernon Hills, IL, USA) was inserted in the right femoral vein, and 5ml/kg normal saline was infused.

At the end of ischaemia time, the abdominal wound was re-opened and reperfusion was performed as described in Chapter 2.

Study A

Fluid resuscitation with 0.9% normal saline was administered through the right femoral vein in animals from all groups with 5ml/kg boluses after 60, 120, and 180min from the beginning of reperfusion.

Survival was then monitored for up to 240min from the onset of reperfusion, and experimental animals were considered dead after 5min of uninterrupted cardiorespiratory arrest. Survival time from the beginning of reperfusion was

recorded, and animals that were still alive at the end of the experiment were killed by exsanguination under terminal anaesthesia.

In a preliminary set of experiments during the setup of the animal model, I attempted to insert an arterial line for continuous measurement of heart rate and blood pressure as described in adult animals (see Chapter 5). After exposing the femoral vessels through a longitudinal incision, a 0.16mm polyimide catheter was inserted into the femoral artery, and connected to a transducer. Unfortunately, the very small internal diameter of the catheter resulted in a high resistance system with unreliable measurements of both blood pressure and heart rate. An additional attempt was made to cannulate the abdominal aorta at the bifurcation with a 26G cannula (BD Neoflon, Helsinborg, Sweden), which resulted in exsanguination of the animals. Invasive measurement of haemodynamics was therefore not performed in the experimental animals of Study A and B.

Study B

Rats were resuscitated with 0.9% normal saline through the right femoral vein with 5ml/kg each after 5 and 60min from the onset of reperfusion.

One min before the end of the experiment, animals were heparinised by infusion of 1,000 IU/kg sodium heparin (Sigma-Aldrich Co., Poole, UK) in 5ml/kg 0.9% saline via the femoral line. Animals were sacrificed by exsanguination under terminal anaesthesia.

6.2.3 Tissue extraction and metabolite measurement

At sacrifice, a liver sample was freeze-clamped in liquid nitrogen for measurements of phosphoenergetics. A blood sample was collected from the thorax, transferred into a heparin tube and centrifuged (3,000rpm for 5min at 4°C), and plasma was stored at -70°C until analysis. Small intestine (10-25cm from the ileocaecal valve) and lungs were also removed and stored at -70°C. Terminal ileum (5 to 10cm from the ileocaecal valve) was fixed in 10% neutral buffered formaldehyde for 24 hours and embedded in paraffin.

Liver phosphoenergetics were quantified by HPLC. Myeloperoxidase activity in ileum and lungs was measured by spectrophotometry. Levels of

malondialdehyde in terminal ileum, lungs and heart were determined by HPLC. The concentration of reduced and oxidised glutathione in ileum was measured by HPLC. Plasma concentration of nitrate plus nitrite, reflecting systemic production of nitric oxide, was quantified by HPLC. Determination of phosphoenergetics, MDA, glutathione, nitrate plus nitrite was performed as described in Chapter 2.

Haematoxylin and eosin staining, and immunostaining for P-Selectin, and ICAM-1 were performed on 5µm sections from paraffin embedded samples of terminal ileum as described in Chapter 2. The degree of histological injury and the level of expression of each adhesion molecule were assessed semi-quantitatively as specified in Chapter 2.

6.2.4 Statistical analysis

Survival from the beginning of reperfusion is expressed as median [range], and results compared by Kaplan-Meier logrank test. Data regarding the severity of histological injury and expression of adhesion molecule in the ileum are expressed as median [range]; comparison between the groups was by Kruskal-Wallis with Dunn's post-test. All other data are expressed as means \pm SEM, and one-way analysis of variance (ANOVA) with Tukey post-test was used for group comparison. Results showing p values <0.05 were considered significant.

6.3 RESULTS

Study A

6.3.1 Survival

Animals receiving sham operation were all still alive after 240min of sham reperfusion, whereas animals undergoing normothermic I/R developed tachycardia and tachypnoea during reperfusion, which became progressively more severe and were followed by death of all experimental rats after 119min from the beginning of reperfusion [range 82-145min] ($p<0.0001$ vs. Control Normothermia, Figure 6.2). However, hypothermia applied at the beginning of reperfusion delayed the development of tachycardia and tachypnoea, and significantly increased survival time to 224min [187-240min]. Interestingly, hypothermic protection persisted in

animals undergoing rewarming after 120min hypothermic reperfusion: no signs of distress developed during rewarming and half of the animals in this group were still alive at the end of the experiment, with a median survival of 233min [169-240min].

Study B

6.3.2 Histological injury in ileum

Samples from all control animals at either normothermia or hypothermia showed completely preserved intestinal architecture, with villi of appropriate length and an intact epithelial layer even at the tip of the villi (Figure 6.3, 6.4 A, 6.4 C). Although tissue injury developed as a consequence of reperfusion in all samples from I/R rats at both normothermia and hypothermia (Figure 3), in both groups disruption was limited to the epithelium and the core of the villi, whereas the crypts, the lamina propria and the submucosa appeared virtually unaffected, and no signs of conspicuous haemorrhage or spontaneous perforation were visible (Figure 6.4 B, 6.4 D).

6.3.3 P-Selectin expression

P-Selectin expression was not detectable in most specimens from normothermic and hypothermic controls, with absent or only weak staining visible on a limited number of vessels in the remaining samples (Figure 6.5, 6.6 A). However, substantial upregulation of P-Selectin was observed following intestinal reperfusion at both normothermia and hypothermia (Figure 6.5): immunostaining was particularly intense in medium-sized vessels of the submucosa, with positive vessels located throughout the specimens (Figure 6.6 B).

6.3.4 ICAM-1 expression

A similar pattern of expression was observed for ICAM-1, with weak constitutive expression on scattered blood vessels of the submucosa in animals subjected to sham operation (Figure 6.7, 6.8 A). Intestinal reperfusion at normothermia caused a marked increase in ICAM-1 expression (Figure 6.7), mostly on medium sized vessels but also on the microcirculation of the submucosa

(Figure 6.8 B, 6.8 C). ICAM-1 expression was similarly intense and similarly distributed following hypothermic reperfusion (Figure 6.7).

6.3.5 Neutrophil infiltration

Intestinal reperfusion at normothermia was characterised by a marked increase in the number of neutrophils infiltrating both the ileum (Figure 6.9 A) and the lungs (Figure 6.9 B) as assessed by myeloperoxidase activity. However, moderate hypothermia applied at reperfusion completely prevented this migration of inflammatory cells not only in the gut, but also in the lungs.

6.3.6 Lipid peroxidation

Concentrations of MDA in plasma, ileum and lungs were significantly higher in I/R rats at normothermia as compared to controls (Figure 6.10), suggesting that reperfusion of the ileum at normothermia greatly enhanced lipid peroxidation of biological membranes. Rescue hypothermia effectively reduced the extent of lipid peroxidation in both lungs (Figure 6.10 C) and plasma (Figure 6.10 A), although the high levels of MDA found in the ileum of hypothermic IR animals (Figure 6.10 B) indicate that lipid peroxidation was unaffected in the gut.

6.3.7 Glutathione content

The depletion of reduced glutathione levels (Figure 6.11 A) together with the decrease in glutathione redox state (Figure 6.11 D) observed in the ileum following I/R at normothermia reflect the loss of reducing equivalents due to the development of oxidative stress associated with normothermic reperfusion. In addition, reduced levels of oxidised (Figure 6.11 B) and total glutathione (Figure 6.11 C) in this group imply irreversible degradation of glutathione caused by high amounts of free radicals produced during reperfusion of the gut (Gibson et al, 1993). Although rescue moderate hypothermia did not affect consumption of the pool of total glutathione, nor it abolished oxidation of reduced glutathione in the intestine, it prevented the drop in glutathione redox state. No differences in glutathione levels or redox state were seen in the lungs (Figure 6.12).

6.3.8 Systemic nitric oxide production

Intestinal I/R at both normothermia and hypothermia induced a similar increase in systemic NO synthesis as measured by plasma nitrate plus nitrite concentration (Figure 6.13). Once again, a significant positive correlation was found between nitrate plus nitrite levels and malondialdehyde in plasma ($r^2=0.1700$; $p=0.0091$, Figure 6.14)

6.3.9 Liver phosphoenergetics

Normothermic intestinal I/R was also associated with a slight (although non-significant) decrease in hepatic ATP levels (Figure 6.15 A), and a marked increase in the concentrations of liver ADP, AMP, and inorganic phosphate (Figure 6.15 B; Figure 6.15 C; Figure 6.15 E respectively), suggesting a reduction in the availability of high energy phosphates. Development of hepatic energy failure was confirmed by the fall in energy charge in the liver following normothermic intestinal I/R (Figure 6.15 F). However, rescue moderate hypothermia prevented the occurrence of hepatic energy failure: in hypothermic I/R rats AMP and inorganic phosphate levels did not increase, and most importantly energy charge was restored to normal levels. Finally, the total concentration of adenine nucleotides was similar in the different groups (Figure 6.15 D), indicating that, as expected, no consumption of the total pool of adenine nucleotides occurred due to either I/R injury or temperature.

6.4 DISCUSSION

Despite the high relevance to paediatric clinical practice, characterisation of intestinal I/R injury in developing animals has been scarcely investigated, and only a few studies have been published regarding therapeutic interventions that could improve outcome in this setting (Chan et al, 2002; Lelli, Jr. et al, 1993). The present study shows that moderate hypothermia significantly improves survival when applied as a rescue at the beginning of reperfusion, and that these benefits are maintained even after rewarming. These findings are particularly interesting in view of potential clinical application, since they suggest that hypothermia could be

advantageous when applied to clinical conditions where the ischaemic insult has already occurred at the time of diagnosis.

The improvement in survival observed in hypothermic animals undergoing rewarming after hypothermic reperfusion is also of significant clinical relevance. Rewarming is a crucial phase which can lead to cardiocirculatory instability, or can cause a rebound systemic inflammatory response (Thoresen et al, 2000). Although very little is known regarding the consequences of rewarming following mesenteric reperfusion, experiments in adult rats have shown that rewarming does not affect outcome when hypothermia is applied from the beginning of ischaemia (see Chapter 5; Kalia et al, 2002b), and I have confirmed these findings in developing rats.

My results also indicate that protection afforded by rescue hypothermia is exerted early during the reperfusion phase, since no additional benefit in survival was observed in rats maintained hypothermic for the whole duration of reperfusion as compared to those who underwent rewarming after 120min of hypothermic reperfusion. Investigation of the early reperfusion phase showed that hypothermia effectively minimised injury to vital organs such as lungs and liver, suggesting that prevention of MODS could account for this improved outcome. Such beneficial effects could derive from direct protection of distant organs, or they may result from the reduction of systemic inflammatory response elicited in the intestine.

The respiratory system is most frequently affected in clinical conditions associated with intestinal I/R in both neonates (Morecroft et al, 1994a; Sonntag et al, 1998) and adults (Harward et al, 1993), and experimental models have shown that development of respiratory failure is closely correlated with mortality following intestinal I/R (Ito et al, 2005). In our infant animal model, mesenteric reperfusion caused both migration of neutrophils and accumulation of lipid peroxidation end-products into the lungs, suggesting the presence of a conspicuous pulmonary inflammatory reaction accompanied by a state of oxidative stress. Application of hypothermia at reperfusion completely abolished sequestration of neutrophils, which is a crucial event in the establishment of lung injury and respiratory failure following intestinal I/R (Ito et al, 2005; Schmeling et al, 1989). Indeed, priming and infiltration of circulating neutrophils into the lungs results in

damage to the pulmonary alveolar capillary bed, with endothelial cell injury and increased vascular permeability (Koike et al, 1995; Schmeling et al, 1989).

The development of oxidative damage to DNA, proteins, and lipids induced by overproduction of reactive oxygen species has also been shown to play a pivotal role in the pathogenesis of pulmonary failure during intestinal reperfusion (Galili et al, 1998), which can be prevented by administration of free radical scavengers such as edaravone (Ito et al, 2005), methylene blue (Galili et al, 1998), and FeTMPyP (Chapter 4). Oxidative stress was prevented by hypothermia in the current study, as suggested by levels of lipid peroxidation end-product MDA. This is more likely to be due to a reduced free radical production rather than to an increase in antioxidant defences, since no difference was observed in pulmonary levels of the major antioxidant glutathione. In fact, inflammatory cells are known to be one of the main sources of reactive oxygen species following reoxygenation of ischaemic tissue (Cuzzocrea et al, 2001b), and the concomitant reduction in pulmonary neutrophil infiltrate could contribute to explain the decreased extent of oxidative stress observed in hypothermic rats.

Interestingly, intestinal concentrations of reduced appeared to be significantly lower in my infant rat model (approximately 150 nmol/g wet weight in normothermic controls) as compared to adult rats (approximately 220 nmol/g wet weight in normothermic controls, see Chapter 5). Glutathione redox state in the ileum was also significantly lower in newborn animals (approximately 0.4 in normothermic control infants vs. 2.7 in normothermic control adults, see Chapter 5). This age-dependent increase of glutathione antioxidant system seems to be supported by the findings of both lower levels of reduced glutathione and glutathione redox state in human plasma from newborns and infants compared to older children and adults (Di Giuseppe et al, 2004). Although the reasons for these differences are unclear, experimental evidence suggests that lower levels of tissue glutathione and glutathione peroxidase activity in newborn rats compared to adults may reflect an enhanced tissue oxidative capacity in adults compared to newborns (Lattari et al, 1997).

Rescue hypothermia did not affect nitrosative stress following normothermic ischaemia in my animal model of neonatal mesenteric reperfusion injury, as

instead is the case for adult animals (see Chapter 5). This may reflect the numerous differences in nitric oxide metabolism and function between the neonatal and adult period (including the different plasma levels of nitrate plus nitrite measured in infant and adult animals) or alternatively it could depend on the different time frame adopted between my adult and neonatal model. Baseline levels of nitrate plus nitrite averaged 50 μ mol/L in control adult rats (see Chapter 5), and more than twice in infant rats in the current experiment. This finding is consistent with the higher production of nitric oxide previously reported in human infants compared to adults, as assessed by measurement of urinary nitrate excretion (Honold et al, 2000). Hepatic failure is a common complication of conditions associated with mesenteric reperfusion, and is observed in up to 44% of neonates with NEC (Sonntag et al, 1998). In my animal model, profound liver energy failure developed in the early phase of normothermic reperfusion of the ileum. This is an early marker of hepatic injury and an accurate predictor of survival in rats with acute liver failure (Corbin et al, 2003). It has been previously shown that therapeutic hypothermia applied from the beginning of mesenteric ischaemia improves survival by preventing hepatic energy failure (Vejchapipat et al, 2001), and this has been confirmed when hypothermia is applied as a rescue at reperfusion in adult rats (Chapter 5). The present study shows that the same protection on liver energy status can be achieved in infant animals when hypothermia is applied during reperfusion only. A reduced energy requirement during hypothermia could explain the maintenance of an adequate energy charge (Jalan et al, 1999), which is crucial in an organ with a high metabolic activity such as the liver (Jaeschke et al, 2003). In addition, preservation of hepatic energy status could result from down-regulation of pro-inflammatory and pro-apoptotic transcription factors (Parkinson et al, 2004) or from maintenance of an adequate hepatic blood inflow during reperfusion (Turnage et al, 1996).

Although hypothermia did not protect from the development of histological injury in the reperfused gut, which was characterised by a profound disruption in tissue architecture, it completely abolished neutrophil infiltration in the ileum. The reperfused intestine is known to act as a priming site for circulating neutrophils; activated neutrophils are then able to migrate through the endothelial barrier and

infiltrate the lung and other distant organs, inducing tissue injury by release of proteolytic enzymes and reactive oxygen species (Koike et al, 1992; Moore et al, 1994). Our findings are consistent with this hypothesis and the prevention of inflammatory cell infiltrate and oxidative stress observed in the lungs could in fact be the consequence of a reduced neutrophil sequestration and priming in the gut, which could also account for the decreased extent of lipid peroxidation in plasma. This is supported by the finding that topical hypothermia applied during mesenteric ischaemia effectively reduces neutrophil priming in the gut in a canine model (Hassoun et al, 2003). Interestingly, this reduction in inflammatory cell infiltrate was not mediated by a down-regulation in expression of endothelial adhesion molecules expression, which is consistent with previous reports (Hill et al, 1992), and may result from a reduced production of chemoattractants or impaired chemotaxis by leukocytes (Fairchild et al, 2000; Kimura et al, 2002).

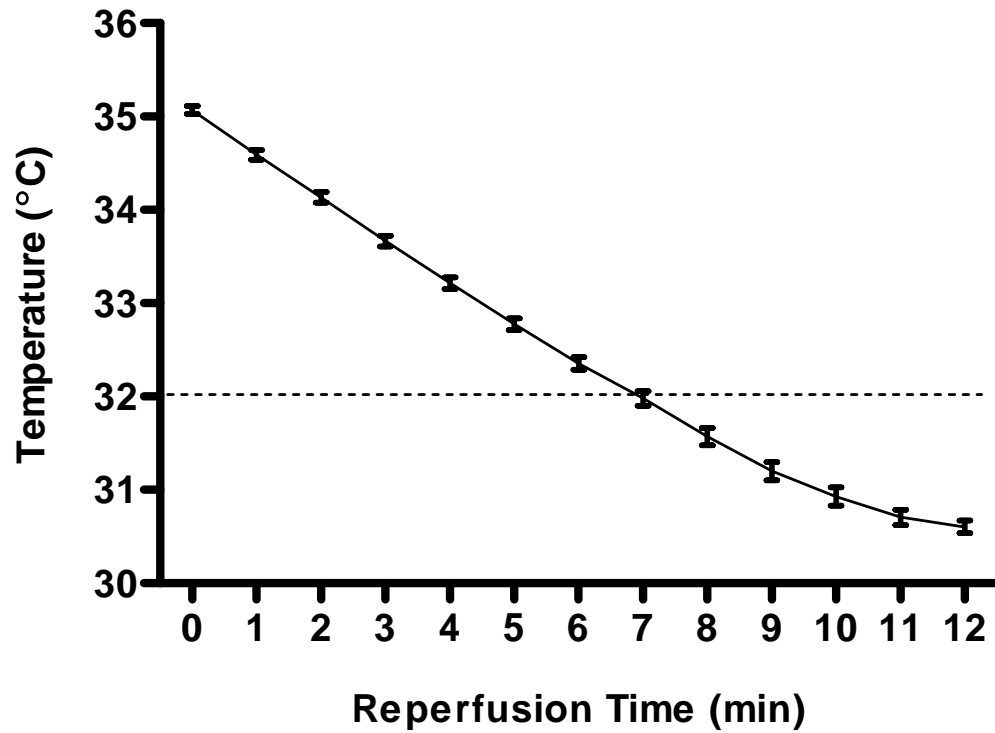
Oxidative stress also developed in the ileum, as proved by the increase in lipid peroxidation and by the concomitant impairment of cellular antioxidant potential, which were not affected by changes in body temperature. Since the number of infiltrating neutrophils was reduced by hypothermia, and their activity is also predicted to be decreased at lower temperatures (Kimura et al, 2002), this state of oxidative stress is likely due to free radicals release by alternative sources including xanthine oxidase (Terada et al, 1992), mitochondria and other intracellular sites (Li et al, 2002).

6.5 CONCLUSIONS

In the current study, I have shown that total-body moderate hypothermia improves survival when applied as a rescue therapy after the end of ischaemia in an infant model of mesenteric reperfusion injury. Moreover, rewarming of hypothermic animals was uneventful and did not hamper the beneficial effects on survival achieved by hypothermic reperfusion. Rescue hypothermia prevented the development of multiple organ failure early during the reperfusion phase by minimising inflammatory cells infiltration in intestine and lungs, oxidative stress in plasma and lungs, and energy failure in the liver. These beneficial effects could

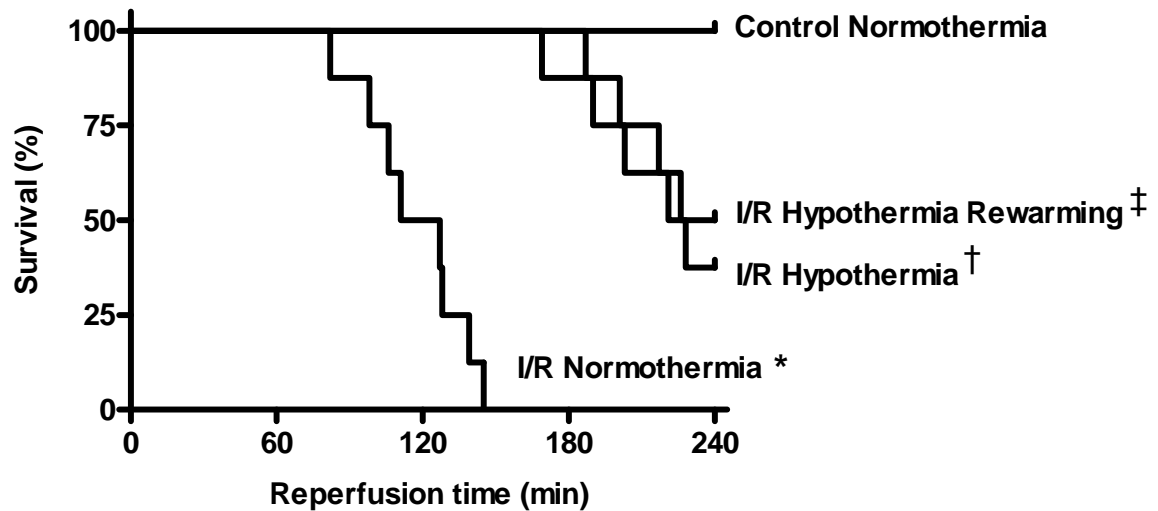
result from direct protection of vital organs such as lungs and liver, and/or from reduced priming of neutrophils in the gut. Our results suggest that therapeutic hypothermia could be beneficial in neonatal conditions characterised by mesenteric ischaemia and reperfusion injury, even when treatment is commenced after the ischaemic injury has been fully established.

Figure 6.1. Core body temperature during the cooling phase.



Core body temperature of rats in the hypothermia groups (n=16) during the cooling phase. Cooling was started at the onset of reperfusion, and target temperature (dotted line) was reached within 12min in all animals. Data are reported as mean \pm SEM.

Figure 6.2. Survival of experimental animals.



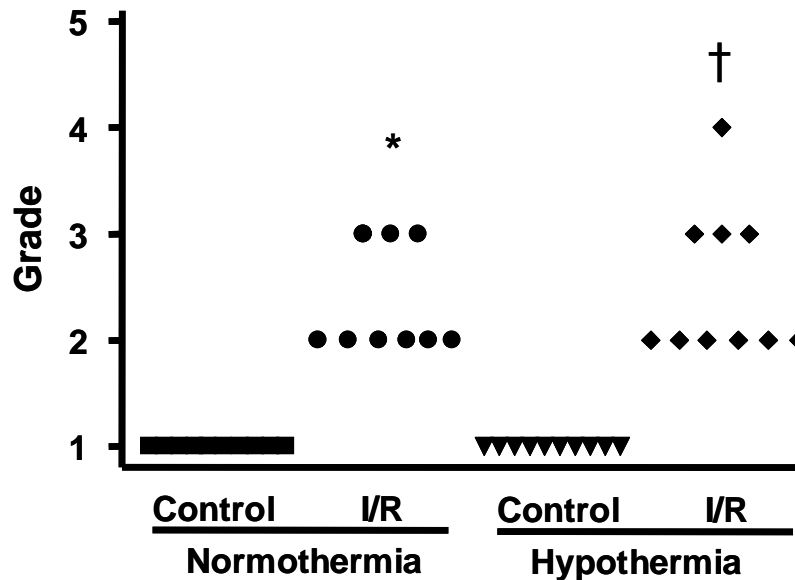
Animals (n=8 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia) with or without rewarming. Survival time is calculated from the beginning of reperfusion; results were compared by Kaplan-Meier logrank test.

* I/R Normothermia: $p < 0.0001$ vs. Control Normothermia; $p < 0.0001$ vs. I/R Hypothermia; $p < 0.0001$ vs. I/R Hypothermia Rewarming.

† I/R Hypothermia: $p = 0.0085$ vs. Control Normothermia.

‡ I/R Hypothermia Rewarming: $p = 0.025$ vs. Control Normothermia.

Figure 6.3. Histological injury in ileum.

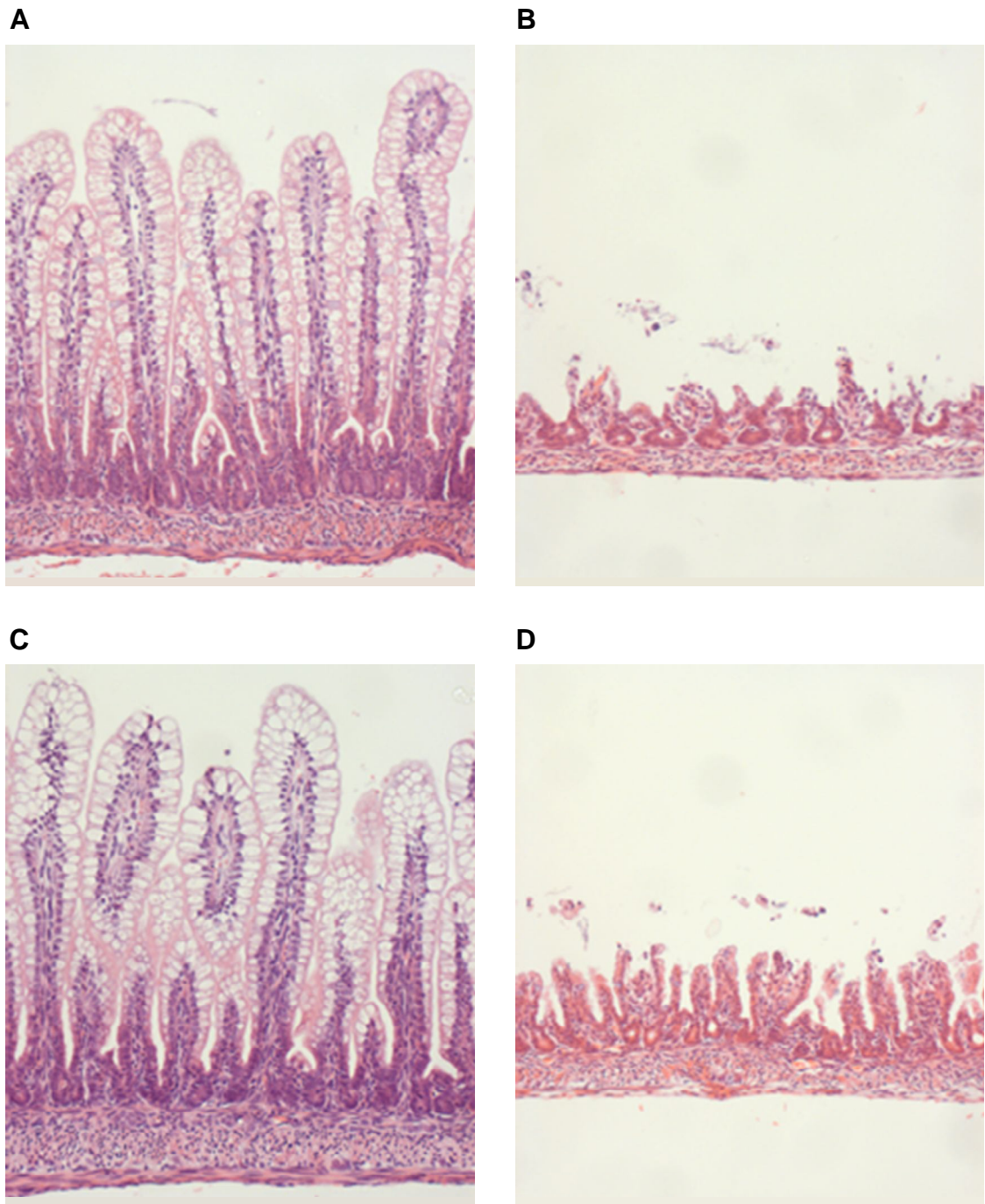


Grade of histological injury in the ileum (5=maximum, 1=minimum). Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia) with or without rewarming. Results were compared using Kruskal-Wallis with Dunn's multiple comparison test.

* I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia.

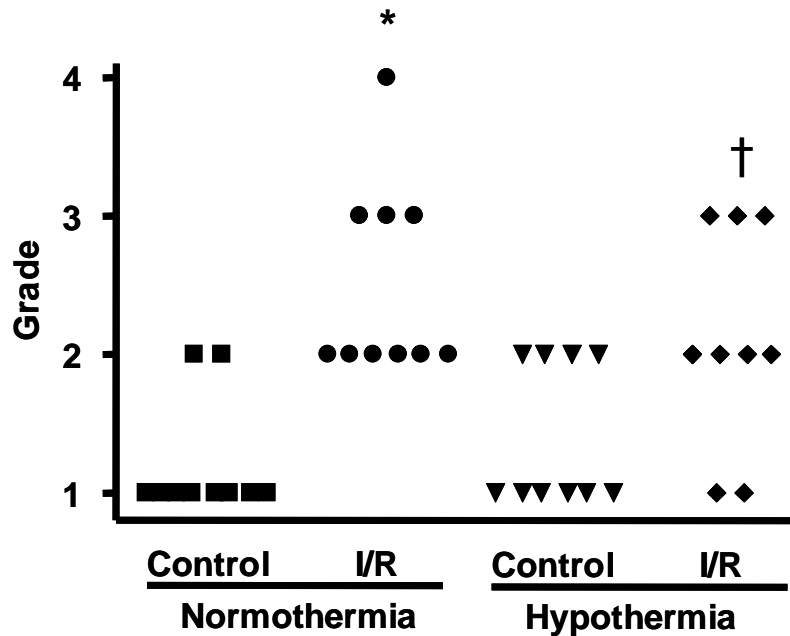
† I/R Hypothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia.

Figure 6.4. Histological appearance of terminal ileum.



Specimens of terminal ileum were stained with haematoxylin and eosin and are from: Control Normothermia [A]; I/R Normothermia [B]; Control Hypothermia [C]; I/R Hypothermia [D]. Magnification is x100.

Figure 6.5. P-Selectin expression in ileum.

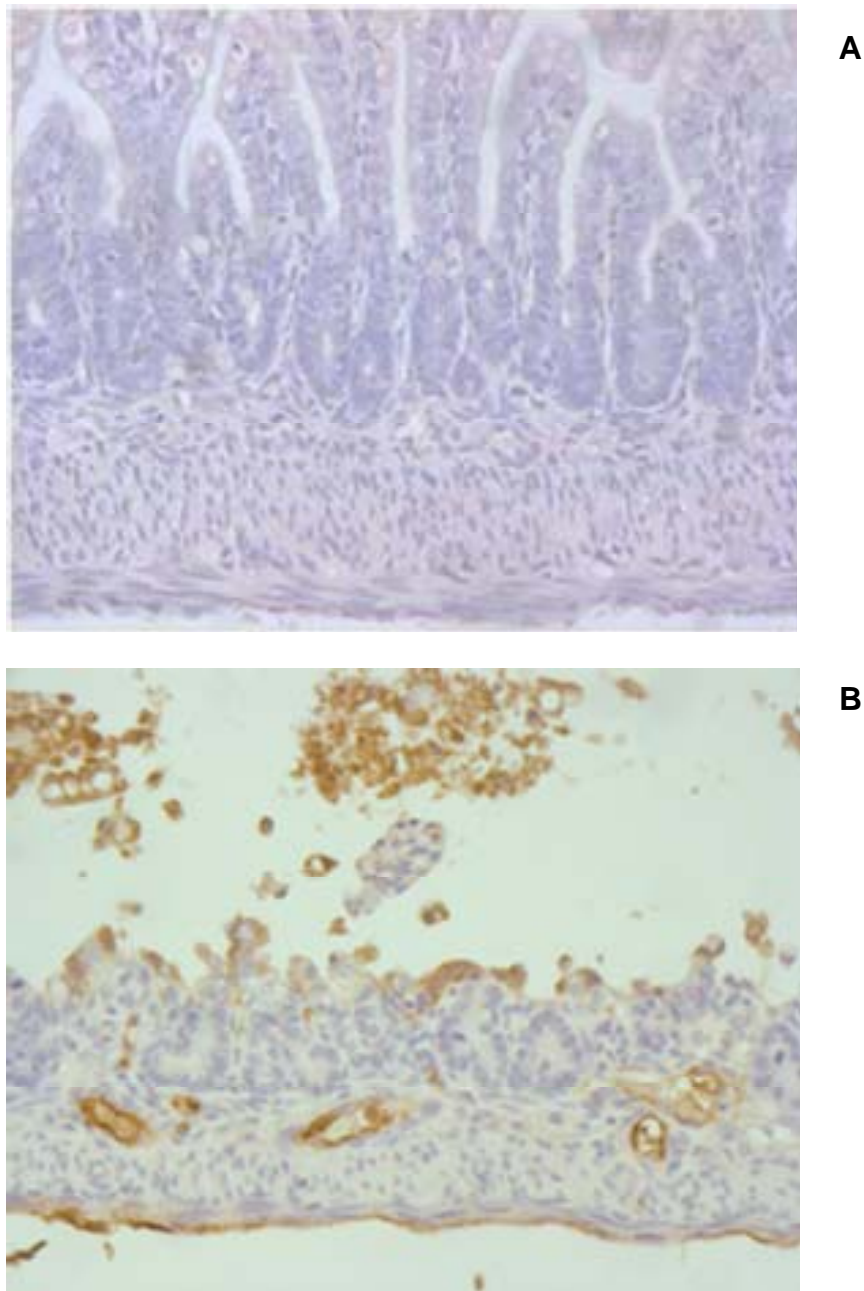


Grade of P-Selectin expression in the ileum. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia) with or without rewarming. Results were compared using Kruskal-Wallis with Dunn's multiple comparison test.

* I/R Normothermia: p<0.01 vs. Control Normothermia; p<0.05 vs. Control Hypothermia.

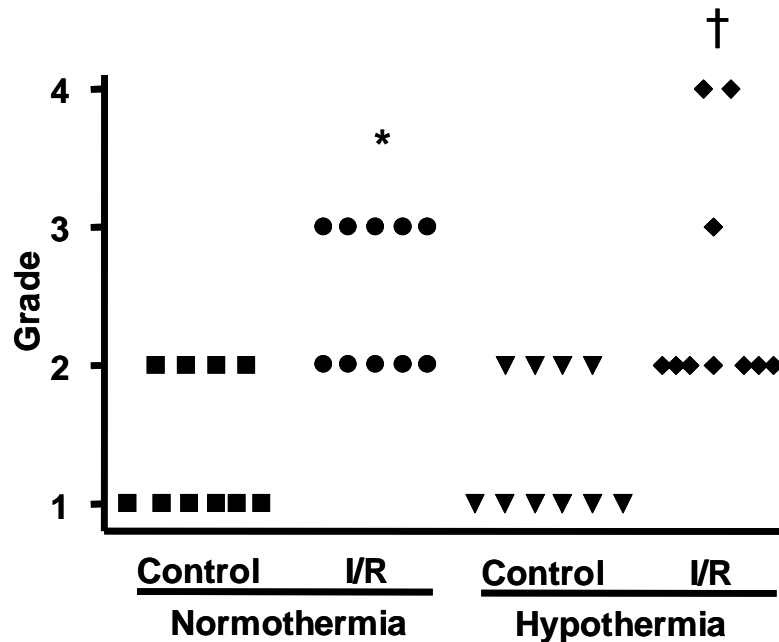
† I/R Hypothermia: p<0.05 vs. Control Normothermia.

Figure 6.6. Histological appearance of P-Selectin expression in ileum.



Specimens of terminal ileum were immunostained for P-Selectin and counterstained with haematoxylin as described in Chapter 2. Specimens are from: Control Normothermia [A]; I/R Normothermia [B]. Magnification is x400.

Figure 6.7. ICAM-1 expression in ileum.

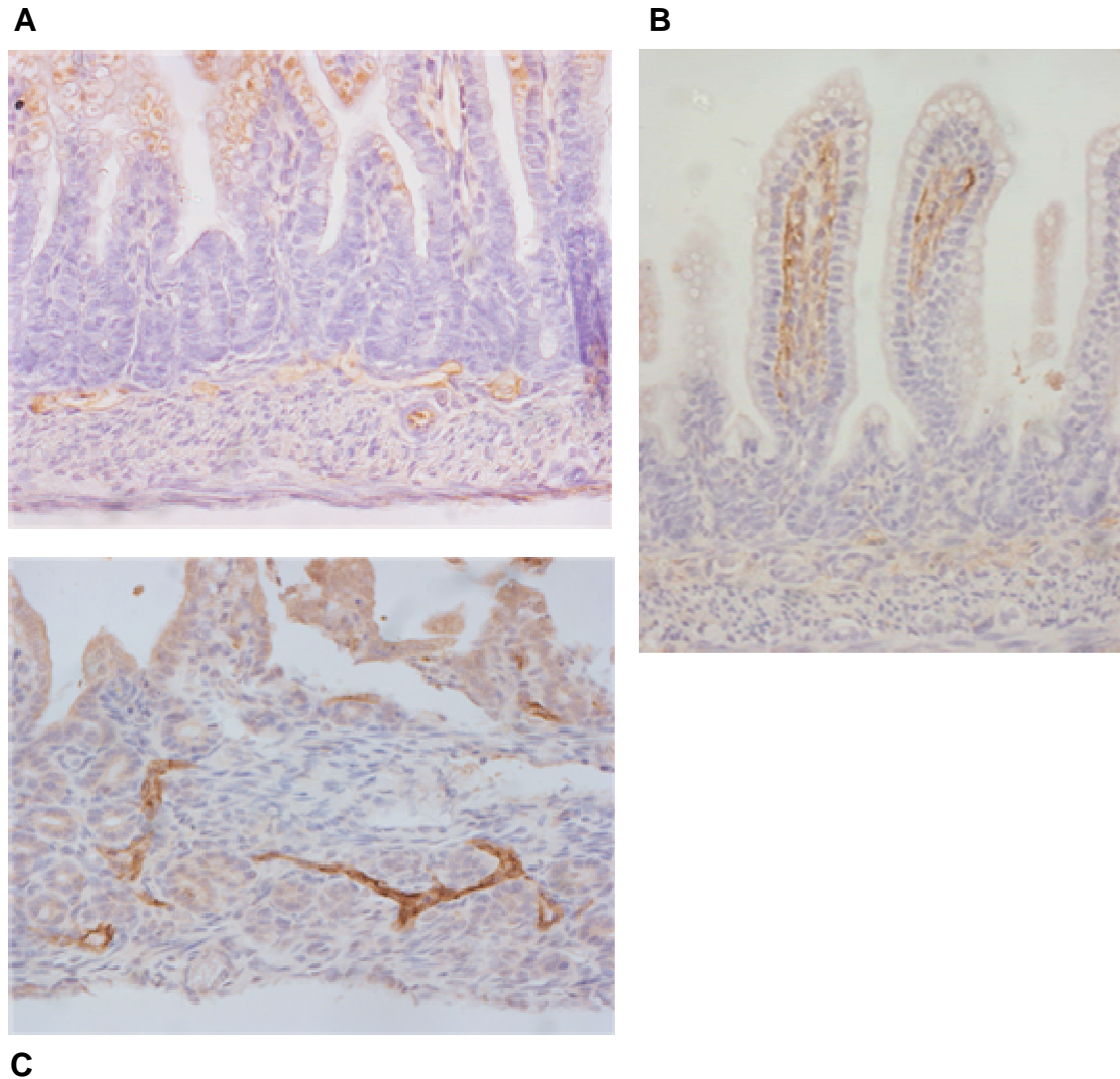


Grade of ICAM-1 expression in the ileum. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia) with or without rewarming. Results were compared using Kruskal-Wallis with Dunn's multiple comparison test.

* I/R Normothermia: $p < 0.01$ vs. Control Normothermia; $p < 0.01$ vs. Control Hypothermia.

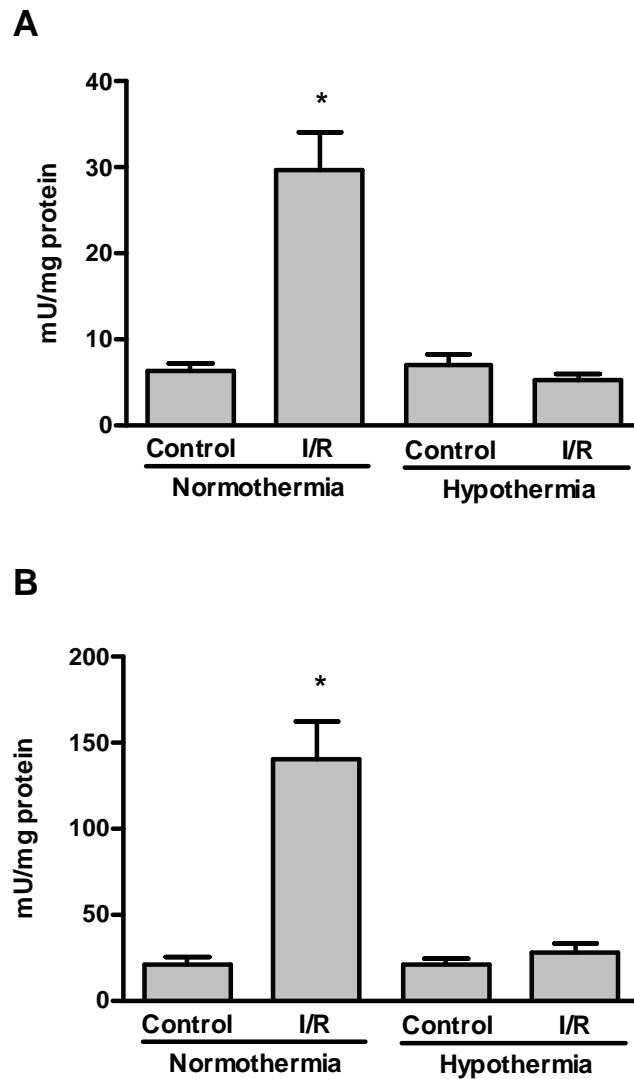
† I/R Hypothermia: $p < 0.05$ vs. Control Normothermia; $p < 0.05$ vs. Control Hypothermia.

Figure 6.8. Histological appearance of ICAM-1 expression in ileum.



Specimens of terminal ileum were immunostained for ICAM-1 and counterstained with haematoxylin as described in Chapter 2. Specimens are from: Control Normothermia [A]; I/R Normothermia [B,C]. Magnification is x400.

Figure 6.9. Neutrophil infiltration in ileum and lungs.

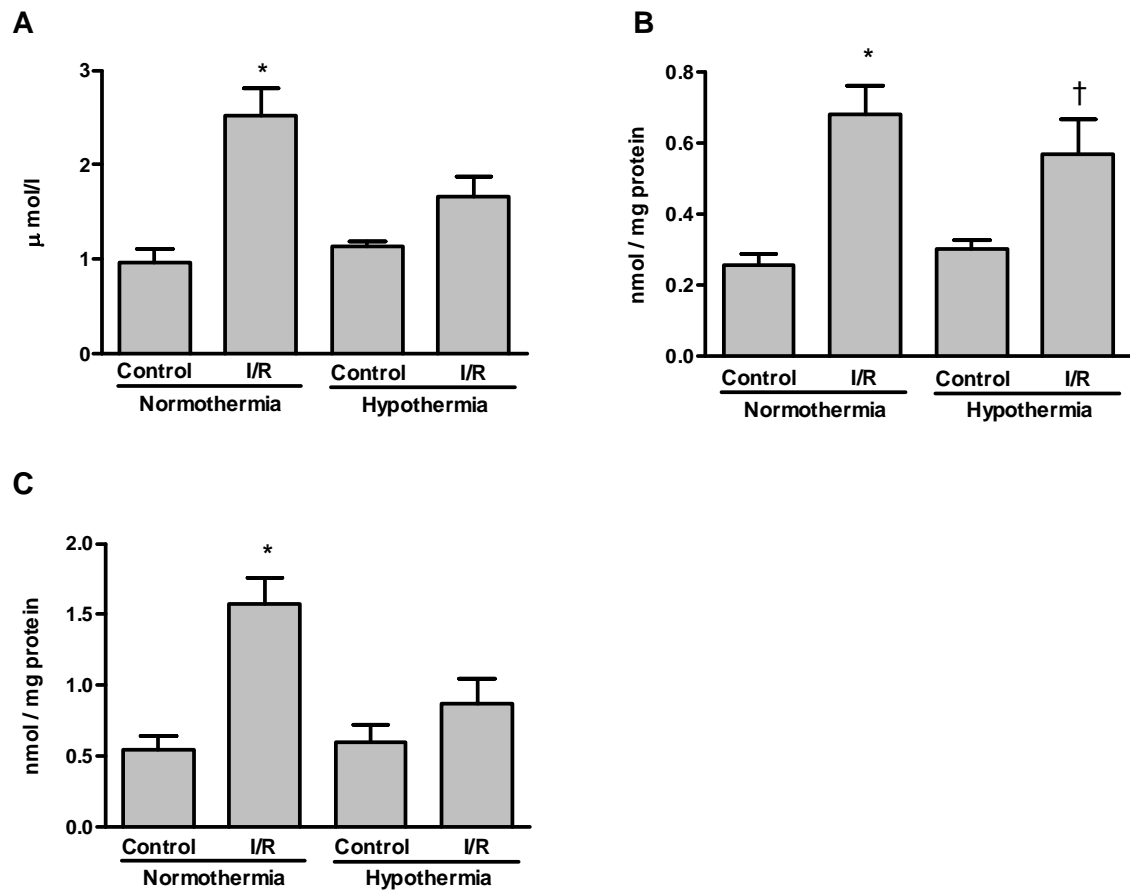


Neutrophil infiltration, assessed as myeloperoxidase activity (MPO) in ileum [A] and lungs [B]. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Results are reported as mean \pm SEM; one-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia; $p < 0.001$ vs. I/R Hypothermia.

[B]: * I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia; $p < 0.001$ vs. I/R Hypothermia.

Figure 6.10. Malondialdehyde concentration in plasma, ileum and lungs.



Concentration of malondialdehyde (MDA) in plasma [A], ileum [B], and lungs [C]. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentration of MDA is expressed as $\mu\text{mol/l}$ for plasma, and as nmol/mg protein for ileum and lungs. Results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

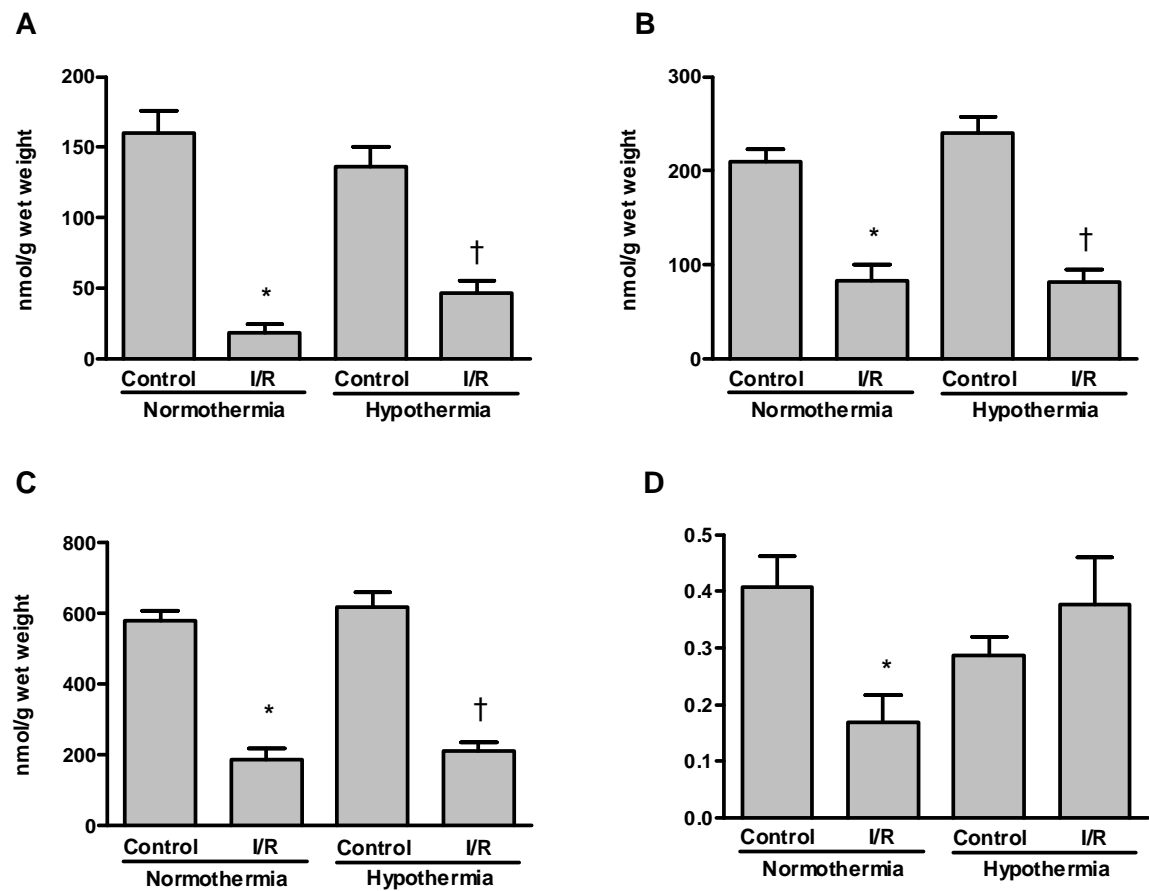
[A]: * I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia; $p = 0.017$ vs. I/R Hypothermia.

[B]: * I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p = 0.001$ vs. Control Hypothermia.

† I/R Hypothermia: $p=0.009$ vs. Control Normothermia; $p=0.032$ vs. Control Hypothermia.

[C]: * I/R Normothermia: $p<0.001$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia; $p=0.009$ vs. I/R Hypothermia.

Figure 6.11. Glutathione concentration in ileum.



Concentration of reduced (GSH) [A], oxidised (GSSG) [B], and total (calculated as $GSH + 2 \times GSSG$) [C] glutathione, and glutathione redox state (calculated as $GSH/2 \times GSSG$) [D] in ileum. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentrations of GSH, GSSG and total glutathione are expressed as nmol/g wet weight. Results are reported as mean \pm SEM; one-way ANOVA with Tukey post hoc test was used for group comparison.

[A]: * I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia.

† I/R Hypothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia.

[B]: * I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia.

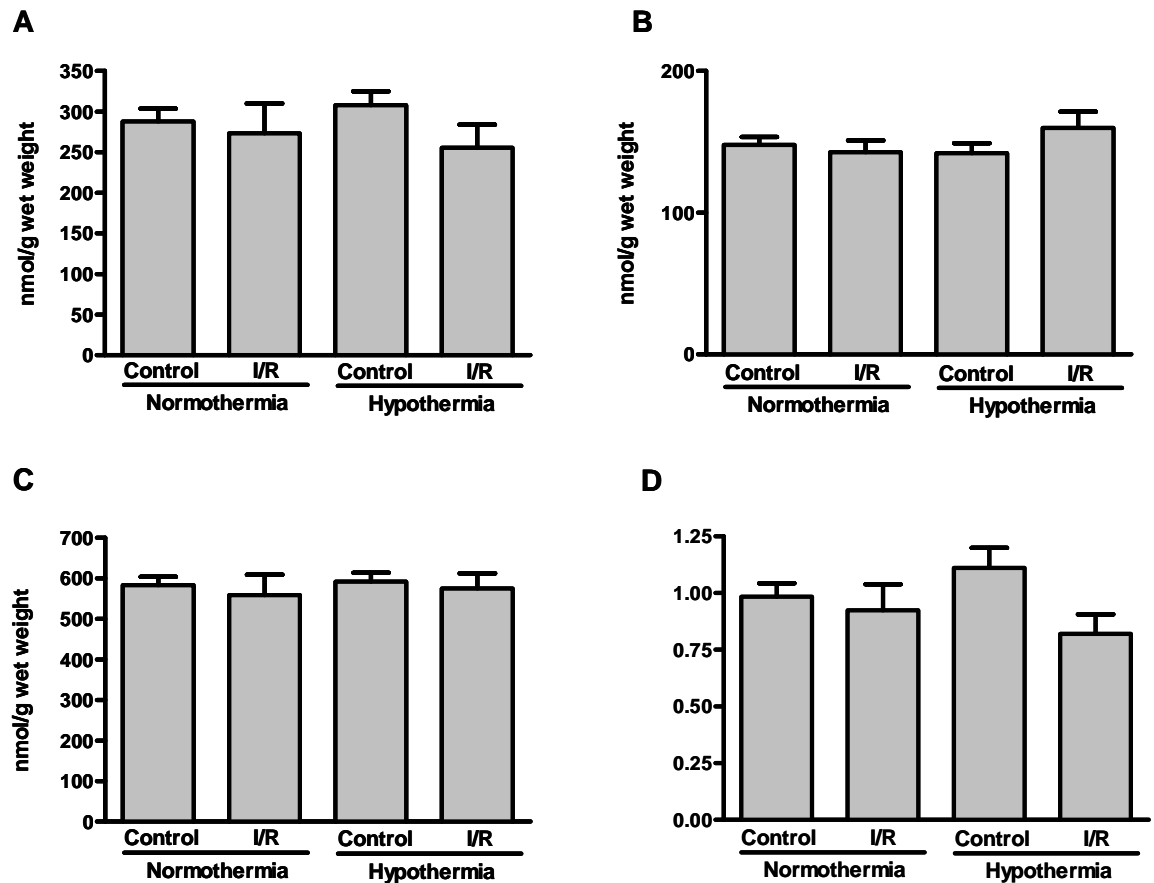
† I/R Hypothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia.

[C]: * I/R Normothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia.

† I/R Hypothermia: $p < 0.001$ vs. Control Normothermia; $p < 0.001$ vs. Control Hypothermia.

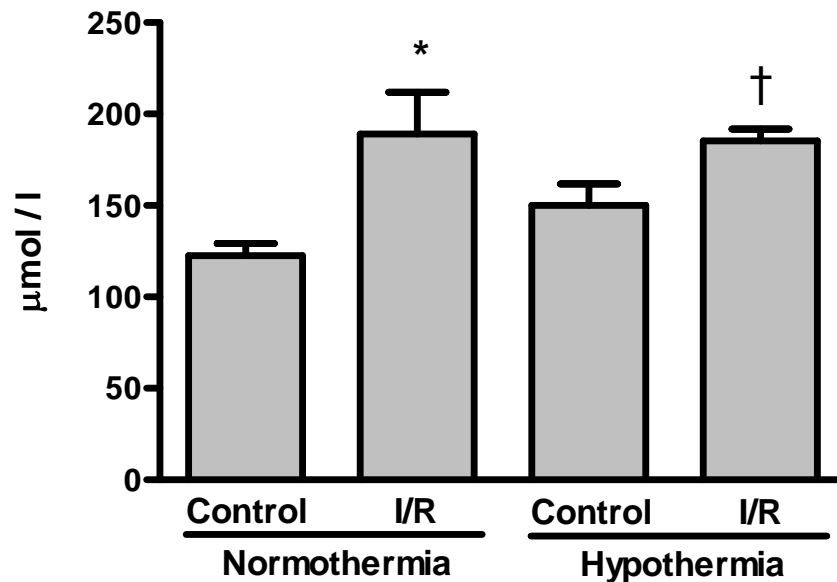
[D]: * I/R Normothermia: $p = 0.028$ vs. Control Normothermia.

Figure 6.12. Glutathione concentration in lungs.



Concentration of reduced (GSH, Figure 16A), oxidised (GSSG, Figure 16B) and total (Figure 16C) glutathione, and glutathione redox state (Figure 16D) in lungs. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentrations of GSH, GSSG and total glutathione are expressed as nmol/g wet weight. Results are reported as mean \pm SEM; one-way ANOVA with Tukey post hoc test was used for group comparison.

Figure 6.13. Concentration of nitrate plus nitrite in plasma.

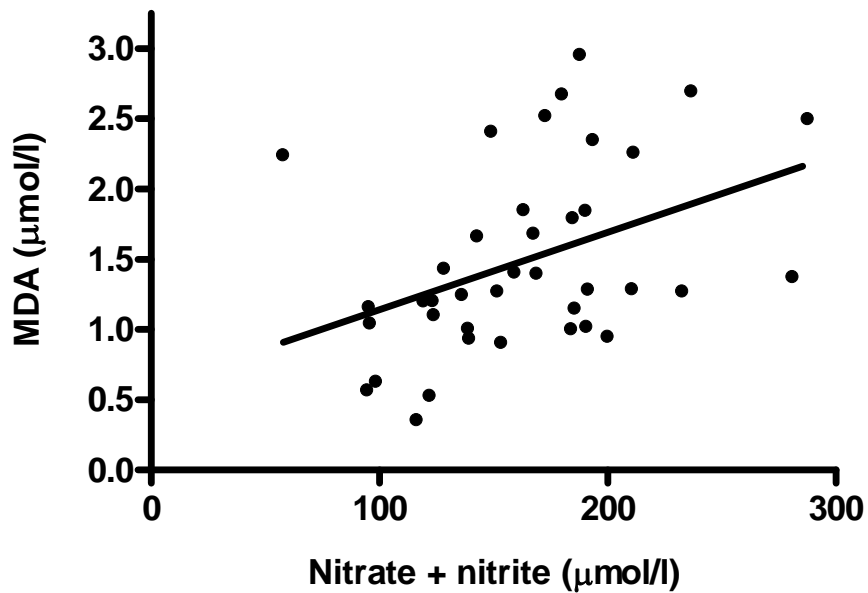


Systemic production of nitric oxide measured as concentration of nitrate plus nitrite in plasma. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R) at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Data are expressed as $\mu\text{mol/l}$ and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

* I/R Normothermia: $p=0.008$ vs. Control Normothermia.

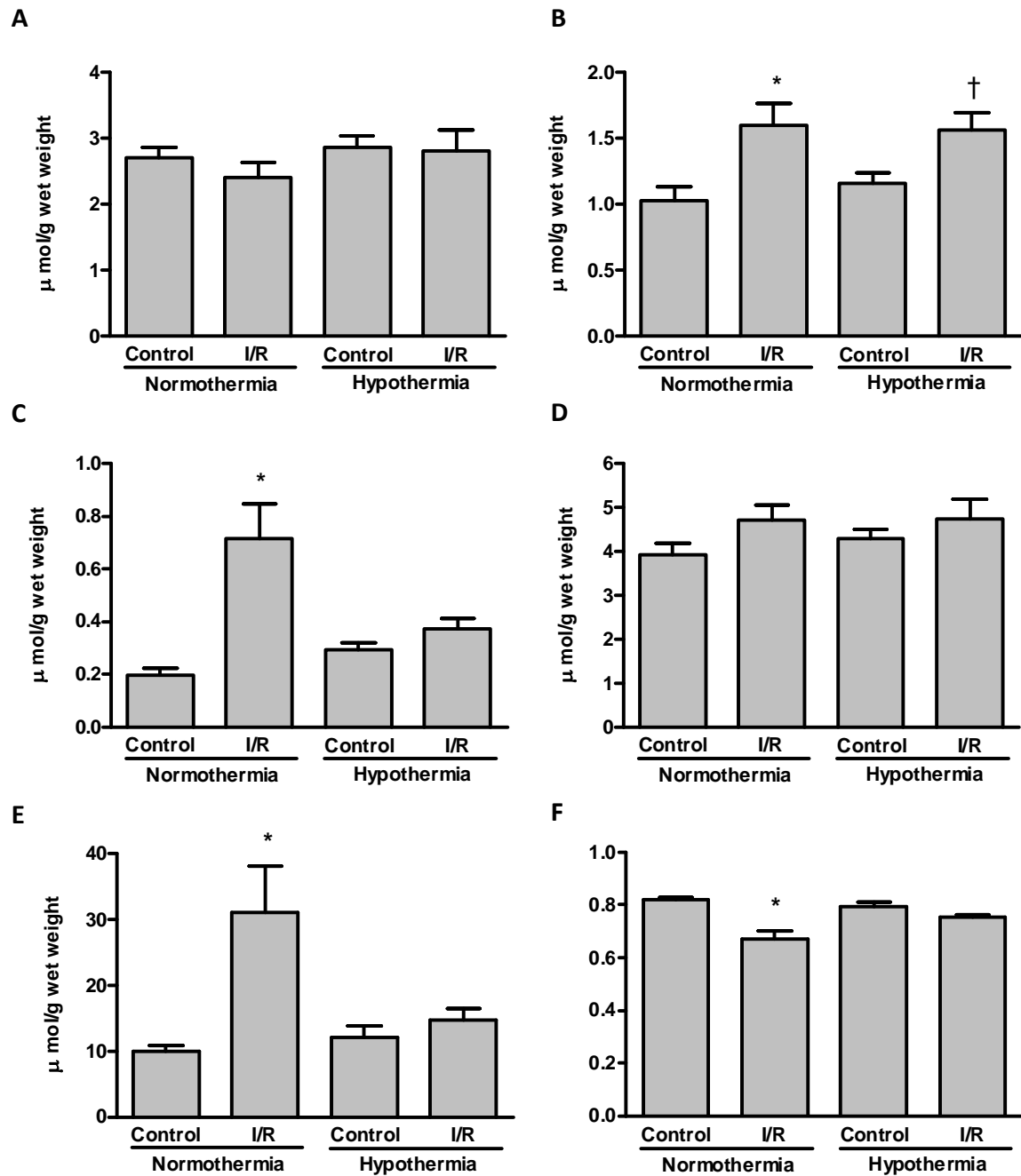
† I/R Hypothermia: $p=0.013$ vs. Control Normothermia.

Figure 6.14. Correlation between nitrate plus nitrite and malondialdehyde concentration in plasma.



Correlation between nitrate plus nitrite (expressed as $\mu\text{mol/l}$) and malondialdehyde (MDA, expressed as $\mu\text{mol/l}$) in plasma after removal of one single outlier (linear regression analysis indicated a correlation with $r^2=0.1700$; $p=0.0091$).

Figure 6.15. Concentration of phosphoenergetics and energy charge in liver.



Concentration of ATP [A], ADP [B], AMP [C], total adenine nucleotides (calculated as ATP+ADP+AMP) [D], and inorganic phosphate [E], and energy charge (calculated as $[(\text{ATP} + \frac{1}{2}\text{ADP}) / (\text{ATP} + \text{ADP} + \text{AMP})]$) [F] in liver. Animals (n=10 per group) underwent sham operation (Control) or intestinal ischaemia and reperfusion (I/R)

at either normothermia (Normothermia) or moderate hypothermia (Hypothermia). Concentrations of ATP, ADP, AMP, total adenine nucleotides, and inorganic phosphate are expressed as $\mu\text{mol/g}$ wet weight and results are reported as mean \pm SEM. One-way ANOVA with Tukey post hoc test was used for group comparison.

[B]: * I/R Normothermia: $p=0.014$ vs. Control Normothermia.

† I/R Hypothermia: $p=0.025$ vs. Control Normothermia.

[C]: * I/R Normothermia: $p<0.001$ vs. Control Normothermia; $p=0.001$ vs. Control Hypothermia; $p=0.008$ vs. I/R Hypothermia.

[E]: * I/R Normothermia: $p=0.002$ vs. Control Normothermia; $p=0.005$ vs. Control Hypothermia; $p=0.019$ vs. I/R Hypothermia.

[F]: * I/R Normothermia: $p<0.001$ vs. Control Normothermia; $p<0.001$ vs. Control Hypothermia; $p=0.016$ vs. I/R Hypothermia.

CHAPTER 7

ADHESION MOLECULE EXPRESSION AND INFLAMMATORY CELL INFILTRATE IN HUMAN NECROTIZING ENTEROCOLITIS

CHAPTER 7

ADHESION MOLECULE EXPRESSION AND INFLAMMATORY CELL INFILTRATE IN HUMAN NECROTIZING ENTEROCOLITIS

7.1 BACKGROUND

Despite advances in preventative strategies and clinical management, NEC remains the most common surgical emergency in newborn infants, accounting for 1 to 3% of admissions to neonatal intensive care units (Neu, 1996) and requiring surgical intervention in up to 63% of cases (Lee et al, 2003; Pierro et al, 2003). Inflammation is a nearly ubiquitous histological finding in NEC, with features of acute inflammation such as infiltration of leukocytes present in the vast majority of intestinal specimens (Ballance et al, 1990).

Although many aspects of the pathogenesis of NEC are still unclear, recent evidence from experimental models of NEC suggests that activation of inflammatory cascade with recruitment of inflammatory cells and production of soluble mediators plays a major role in promoting tissue injury in the intestine (Caplan et al, 1990; Ng et al, 2003; Kozol, 1992; Ford et al, 1996). In particular, migration and activation of neutrophils and macrophages is believed to be one of the main pathways leading to mucosal and submucosal degradation in NEC (Pender et al, 2003). This is confirmed by the findings that inhibition of neutrophils by pre-treatment with vinblastine (Museumche et al, 1991), and of macrophages by semapimod (Zamora et al, 2005) reduced the extent of intestinal injury and the severity of disease in animal models of NEC.

However, the pattern of inflammatory cell infiltrate in the intestine has been poorly investigated in human NEC, and the mechanisms regulating their recruitment and activation is largely unknown. In particular, no studies are reported in the English literature regarding the role of endothelial adhesion molecules P-Selectin, ICAM-1, or E-Selectin in NEC.

P-selectin, an adhesion molecule expressed by activated endothelial cells and platelets, mediates the early phases of leukocyte adherence to the endothelium. Expression of P-selectin has been shown to be crucial to leukocyte recruitment in

many human inflammatory processes (Bevilacqua et al, 1994) as well as in animal models of intestinal ischaemia and reperfusion, intestinal transplantation and sepsis (Hayward et al, 1999; Davenpeck et al, 1994; Carmody et al, 2004; Riaz et al, 2002; Farmer et al, 2002; Opal et al, 2001). E-Selectin expression on endothelial cells involves transcriptional mechanisms (Bevilacqua et al, 1989), and mediates the transition from slow rolling to firm leukocyte arrest (Smith et al, 2004). Blockade of E-Selectin prevents leukocyte infiltration and reduces tissue injury in experimental models of myocardial and cerebral ischaemia (Carter et al, 2002; Huang et al, 2000; Carter et al, 2000), and reduces reperfusion-induced neutrophil recruitment in the intestine (Russell et al, 2000). ICAM-1 mediates firm adhesion of leukocytes to, as well as migration through, endothelial cells (Schenkel et al, 2004), and represents an essential step in leukocyte infiltration into different organs in experimental sepsis or mesenteric ischaemia and reperfusion (Raeburn et al, 2002; Beck-Schimmer et al, 2002; Ilhan et al, 2003).

The aim of the present study was to evaluate the expression of endothelial adhesion molecules in intestinal specimens from neonates with NEC, and to investigate their role in the development of inflammation and tissue injury.

7.2 MATERIALS AND METHODS

The study protocol was approved by the Great Ormond Street Hospital for Children and Institute of Child Health Research Ethics Committee.

7.2.1 Patients

A retrospective review of the neonatal intensive care register was performed and infants who had undergone bowel resection between September 2000 and April 2002 were identified. Histopathology records were also reviewed to identify those diagnosed with necrotizing enterocolitis and those with other conditions.

A senior consultant pathologist selected representative specimens from small and large bowel of 13 of the NEC patients and 7 controls from neonates with non-inflammatory conditions (ileal atresia: n=5; ileal stenosis: n=1; anorectal malformation: n=1), and tissue sections were obtained from paraffin embedded

blocks. In 9 NEC patients sections were taken from both severely affected areas and resection margins, providing 22 sections from 13 patients. In control patients, one section per individual was evaluated. Demographics and information regarding clinical conditions at the time of surgery were retrieved from the patients' records by a consultant intensivist, and are shown in Table 8.1. Data relative to blood tests (full blood count and coagulation study) immediately before surgery, also retrieved by a consultant intensivist, are shown in Table 8.2.

7.2.2 Histological injury

Intestinal specimens were fixed in 10% formaldehyde buffered solution for 24 h followed by paraffin-embedding, and tissue sections (4µm thick) were cut. Following dewaxing, sections were stained with haematoxylin and eosin for histological evaluation.

To evaluate the severity of histological injury, haematoxylin and eosin stained sections of NEC and control patients were scored using a semi-quantitative scale adapted from Ade-Ajayi (Ade-Ajayi et al, 1996):

- Grade 1: Normal (no inflammation)
- Grade 2: Mild NEC (inflammatory changes confined to the mucosa)
- Grade 3: Moderate NEC (changes affecting the mucosa and submucosa)
- Grade 4: Severe NEC (inflammation involving mucosa, submucosa and serosa)
- Grade 5: Very severe NEC (transmural necrosis including perforation)

Three control samples were considered 'normal' by all investigators and served as references for comparison to the remaining sections, which were scored independently in a blinded fashion by myself and another observer. Quality control was achieved by assessment of random samples by two additional investigators.

7.2.3 Polymorphonuclear cells infiltration

Immunostaining of 4µm paraffin embedded sections for neutrophil elastase was used to detect infiltration of polymorphonuclear cells into intestinal tissue.

After blocking endogenous peroxidase by incubation in 3% H₂O₂ in PBS for 20min, primary antibody (neutrophil elastase, DakoCytomation, Cambridge, UK;

1:1000 dilution in PBS) was incubated for 30min, and secondary incubation with labelled polymer HRP (EnVision, DakoCytomation, Cambridge, UK) was subsequently allowed for 30min. Sections were stained with DAB before counterstaining with haematoxylin.

Neutrophil infiltrate in the mucosa was quantified by measuring the number of neutrophil elastase positive cells. Tissue sections were evaluated blindly. For each specimen, neutrophil elastase positive cells on 20 high power fields (x400 magnification) representative of the whole section were counted, and the mean was calculated. The degree of neutrophil infiltration in the mucosa was expressed on a five-point scale:

- Grade 1: 0-10 positive cells per field
- Grade 2: 11-25 positive cells per field
- Grade 3: 26-100 positive cells per field
- Grade 4: 101-200 positive cells per field
- Grade 5: >200 positive cells per field

7.2.4 Macrophage infiltration

Infiltration of macrophages in intestinal tissue was assessed by immunostaining of 4µm paraffin embedded sections for pan-macrophage marker CD68 (cluster of differentiation 68).

After antigen retrieval, achieved by microwaving for 10min in 1% citrate buffer, specimens were incubated with 3% H₂O₂ for 20min, and with primary mouse anti-human CD68 (PG-M1, DakoCytomation, Cambridge, UK; 1:200 dilution) for 30min. Following secondary incubation with labelled polymer HRP, sections were stained with DAB and counterstained with haematoxylin.

The extent of macrophage infiltration was quantified by measuring the number of CD68 positive cells in tissue. Specimens were assessed in a blinded fashion. For each specimen, the number of CD68 positive cells in 20 high power fields (x400 magnification) in the mucosa, submucosa and serosa were counted, and the average was calculated.

7.2.5 Adhesion molecule expression

Immunostaining for CD62P was performed to identify P-selectin expression, and double staining for CD62P and CD41 (cluster of differentiation 41) allowed differential evaluation of P-selectin expression on platelets and endothelial cells; ICAM-1 expression was identified by immunostaining for CD54; immunohistochemistry for CD62E allowed recognition of E-Selectin expression.

P-Selectin (CD62P). Antigen retrieval was achieved by heating the tissue sections in 1% citrate buffer (pH 6.0) for 10min. After blockade of endogenous peroxidase activity, incubation with 10% normal goat serum was performed to minimise nonspecific binding. Primary anti-human CD62-P (P-selectin) antibody (BD Biosciences, San Jose, CA, USA; 1:200 dilution in PBS) was incubated overnight at 4°C. Following secondary incubation with labelled polymer HRP, sections were stained with DAB and counterstained with haematoxylin.

The expression of P-selectin was assessed by grading the intensity of ring-shaped vessels expressing CD62P on DAB stained sections. The specimens were scored independently and blindly by myself and two other investigators. P-selectin expression on large and medium-sized vessels and on the microcirculation were evaluated separately. Intensity of the staining was expressed on a semi-quantitative scale:

- Grade 1: Weak staining (0-10% of vessels stained)
- Grade 2: Mild staining (10-25% of vessels stained)
- Grade 3: Moderate staining (25-50% of vessels stained)
- Grade 4: Strong staining (50-75% of vessels stained)
- Grade 5: Very strong staining (75-100% of vessels stained)

Dual staining for P-Selectin (CD62P) and CD41. A representative sample of specimens from NEC patients (n=12) was processed with dual staining for CD41-CD62P in order to assess the selective expression of P-selectin on endothelial cells or on platelets. The sections were digested in 0.02% protease at 37°C for 10min for antigen retrieval of CD41. Tissue sections were incubated with 10% normal goat serum, and subsequently with primary anti-CD41 antibody (DakoCytomation,

Cambridge, UK; 1:50 dilution in PBS) for 30 min. Sections were incubated with labelled polymer HRP and stained with DAB. After incubation with levamisole for 5min, primary anti-human CD62-P (P-selectin) antibody (1:200 dilution in PBS) was applied overnight at 4°C. Following incubation with labelled polymer alkaline phosphatase (AP, DakoCytomation, Cambridge, UK), staining with fast red and counterstaining with haematoxylin were performed.

Vessels expressing P-selectin on endothelial cells had a pink coloured, ring-shaped appearance, whereas a brown-orange granular staining filling the lumen of a blood vessel corresponded to activated platelets coexpressing both CD41 and CD62P (Figure 7.13).

ICAM-1 (CD54). Antigen retrieval was by microwaving for 10min in 1% citrate buffer solution. Slides were incubated with 3% H₂O₂ for 20min, and then with 10% normal rabbit serum (DakoCytomation, Cambridge, UK). Primary anti-human ICAM-1 (CD54, R&D Systems, Ltd., Abingdon, UK, 1:200 dilution) was applied for 30min, followed by incubation with biotinylated anti-goat secondary antibody (Vectastain ABC kit, Vector Laboratories, Ltd., Peterborough, UK). Sections were stained with DAB before counterstaining with haematoxylin.

Expression of ICAM-1 on the endothelium was evaluated separately in the mucosa, submucosa and serosa. Sections were scored independently and blindly by myself and two other investigators. Intensity of the staining was expressed on a semi-quantitative scale:

- Grade 1: Weak staining (0-10% of vessels stained)
- Grade 2: Mild staining (10-25% of vessels stained)
- Grade 3: Moderate staining (25-50% of vessels stained)
- Grade 4: Strong staining (50-75% of vessels stained)
- Grade 5: Very strong staining (75-100% of vessels stained)

E-Selectin (CD62E). After microwaving for 10min in 1% citrate buffer solution, 3% H₂O₂ was applied for 20min, followed by 10% normal rabbit serum. Slides were then incubated with goat anti-human E-selectin (CD62E, R&D Systems, Ltd., Abingdon, UK, 1:180 dilution) for 60min, followed by biotinylated anti-goat

secondary antibody (Vectastain ABC kit, Vector Laboratories, Ltd., Peterborough, UK). Sections were stained with DAB and counterstained with haematoxylin.

Unlike P-Selectin and ICAM-1, E-Selectin expression was limited to few specimens from NEC patients, and was absent on the remaining sections from NEC patients, and from controls (see paragraph 8.3.6). No grading system could therefore be applied, and staining for E-selectin was classified as present or absent.

Dual staining for P-Selectin (CD62P) and CD68. Dual staining for P-Selectin and pan-macrophage marker CD68 was performed in order to identify macrophages adherent to blood vessels expressing P-Selectin.

Antigen retrieval was achieved by microwaving tissue sections in 1% citrate buffer for 10min. 3% H₂O₂ was then applied for 20min, followed by 10% normal rabbit serum to minimise nonspecific binding. Primary anti-human CD62-P (P-selectin) antibody (1:200 dilution in PBS) was incubated overnight at 4°C. After secondary incubation with labelled polymer HRP, sections were stained with DAB. Specimens were then incubated with levamisole for 5min, and with primary mouse anti-human CD68 (1:200 dilution) for 30min. Labelled polymer AP was then added, followed by staining with fast red and counterstaining with haematoxylin.

Dual staining for ICAM-1 (CD54) and CD68. Dual staining for ICAM-1 and pan-macrophage marker CD68 allowed identification of macrophages adherent to blood vessels expressing ICAM-1.

Antigen retrieval was by microwaving for 10min in 1% citrate buffer solution. Slides were incubated with 3% H₂O₂ for 20min, and then with 10% normal rabbit serum. Primary anti-human ICAM-1 (CD54, 1:200 dilution) was applied for 30min, followed by incubation with biotinylated anti-goat secondary antibody and staining with DAB. After incubation with levamisole for 5min, primary mouse anti-human CD68 (1:200 dilution) was applied for 30min. Specimens were then incubated with labelled polymer AP, stained with fast red and counterstained with haematoxylin.

7.2.6 Statistical analysis

Results are expressed as median [interquartile range]. Mann-Whitney U test and Fisher's exact test were used for group comparison as appropriate. Spearman rank test was used to investigate the correlation between the degree of expression of adhesion molecules, and leukocyte infiltration. A model of linear regression was used to assess the influence of leukocyte infiltration and adhesion molecule expression, and the degree of histological injury as well as clinical outcome (number of organs in failure, development of sepsis, and survival). Before a model of linear regression was built, correlation between single variables was investigated by Spearman rank test. The expression of P-Selectin on the microcirculation and on large calibre vessels were excluded from the model because of significant multi-collinearity ($R^2 > 0.9$). Differences were considered statistically significant for p values < 0.05 .

7.3 RESULTS

7.3.1 Histological injury

The degree of histological injury was significantly higher in sections from NEC patients as compared to control patients (Figure 7.1). Various degrees of histological injury were present in specimens of NEC patients, with most NEC patients presenting features of severe to very severe histological injury on haematoxylin and eosin of at least one stained section (Figure 7.2 C, D). Only three NEC patients showed mildly to moderately affected intestine (Figure 7.2 B). Microscopic appearance of the intestine was unaffected in the majority of control patients (Figure 7.2 A), although in three specimens there were mild features of inflammation.

7.3.2 Polymorphonuclear cell infiltration

Neutrophil infiltration in the mucosa was also much higher in NEC patients than in controls (Figure 7.3). Most sections from NEC patients showed a diffused infiltration of polymorphonuclear cells in the mucosa with over 100 neutrophils per high power field (Figure 7.4 C, D). Accumulation of neutrophils was more evident

in the most damaged areas, where conspicuous sloughing of the mucosal layer and ulceration or exposure of the submucosa with vessel congestion were present. There was a strong positive correlation ($p < 0.001$, $r = 0.644$) between the severity of histological injury and neutrophil infiltration in the mucosa.

7.3.3 Macrophage infiltration

Very few macrophages were visible in the mucosa, submucosa, and serosa of control patients, with none of the sections showing more than 15 CD68 positive cells per HPF (Figure 7.5). However, specimens from NEC patients presented a significantly higher number of infiltrating macrophages scattered in the mucosa and submucosa (Figure 7.6 B, 7.7 B). The pattern of macrophage infiltration was somehow different in the serosa, with clusters of CD68 positive cells located in adipose tissue surrounding large arteries and veins ($p = 0.003$ vs. controls, Figure 7.8 B).

7.3.4 P-selectin expression

Dual staining for CD41 and P-selectin allowed differentiation of P-selectin expression between endothelial cells and platelets. Vessels expressing P-selectin on endothelial cells had a pink coloured, ring-shaped appearance, whereas a brown-orange granular staining filling the lumen of a blood vessel corresponded to activated platelets co-expressing both CD41 and CD62P. In sections from control patients, expression of P-selectin was limited to the endothelium, with no evidence of staining on platelets. Specimens of NEC patients also showed a predominant expression on the endothelium. However, in several sections from NEC patients, aggregates of activated platelets co-expressing CD41 and P-selectin were present in a minority of medium and small vessels, particularly in areas characterised by severe tissue injury and necrosis (Figure 7.13).

P-selectin expression was most evident on endothelium of large vessels located in the submucosa and subserosa, with intense staining on a significant proportion of veins and arteries of both NEC and control patients (Figure 7.9 A, 7.10). A high expression was observed also on medium-sized vessels of many NEC patients, whereas staining in control patients was weaker and limited to a

minority of the medium-sized vessels (Figure 7.9 B, 7.11). Expression of P-selectin was overall weaker on the microcirculation, but the number of positive vessels was significantly higher in NEC patients than in control patients (Figure 7.9 C, 7.12). In addition, expression of P-selectin on medium-sized vessels and on the microcirculation in specimens from control patients was limited to the mucosa and submucosa, whereas in NEC patients with strong or very strong expression of P-selectin a diffuse staining was noticed on blood vessels of all layers, including the muscle.

Expression of P-selectin was also noticed to be particularly high in specimens with severe histological damage. However, in sections with severe histological injury P-selectin positive vessels were particularly abundant in areas with features of strong active inflammation but with no evidence of advanced tissue damage or necrosis (Figure 7.16 B). On the contrary, in adjacent areas where mucosal and submucosal layers were disrupted or frankly necrotic staining for P-selectin was significantly weaker (Figure 7.16 A).

A high number of leukocytes were observed inside blood vessels in sections from NEC patients. In these patients, evaluation of fast-red stained sections on high-power magnification revealed the presence of numerous leukocytes, particularly polymorphonuclear cells, adhering to the endothelium of vessels expressing P-selectin (Figure 7.14). The amount of neutrophils infiltrating the mucosa was strongly correlated with the degree of P-selectin expression on both medium-sized vessels ($p=0.004$, $r=0.513$) and the microcirculation ($p=0.001$, $r=0.578$), but not on large vessels ($p=0.31$). Analysis of sections stained for P-Selectin and CD68 under high-power magnification showed that macrophages were also adhering to P-Selectin expressing blood vessels, particularly small and medium calibre veins (Figure 7.15). However, no significant correlation was found between the degree of expression of P-Selectin on large, medium-sized vessels, or the microcirculation, and the number of macrophages infiltrating the mucosa, submucosa, or serosa.

Also, P-Selectin expression on medium-sized vessels did not significantly influence the degree of histological injury, or the development of clinical sepsis,

the severity of organ failure, or survival when a multiple regression model was applied.

7.3.5 ICAM-1 expression

Specimens from control patients showed very limited staining for ICAM-1 on blood vessels located in the mucosal and serosal layers, whereas a slightly higher level of expression was observed in medium-sized and large vessels of the submucosa (Figure 7.19). Although expression of ICAM-1 was overall higher on small mucosal vessels of NEC patients as compared to controls (Figure 7.17 A), the pattern of expression varied from diffuse staining of more than 75% of blood vessels in some specimen, to weak staining with only few positive vessels in others (Figure 7.18). A similar variability was observed in the serosa of specimens from NEC patients, although the great majority of serosal ICAM-1 positive vessels were large or medium-sized arteries and veins, with limited staining on the microcirculation ($p=0.015$ vs. controls, Figure 7.17 C, 7.20 B). In specimens from NEC patients, staining for ICAM-1 was highest in the submucosa, particularly on large and medium-sized vessels ($p=0.010$ vs. controls, Figure 7.17 B, 7.19 B). Interestingly, ICAM-1 positive vessels located not only in connective tissue but also in the muscle.

A high number of leukocytes was seen adhering to several ICAM-1 positive vessels, mostly veins of small and medium calibre located in the submucosa (Figure 7.21). While several of these adherent leukocytes presented features suggestive for polymorphonuclear (neutrophil) granulocytes, numerous others were identified as macrophages after staining for CD68 (Figure 7.22). These observations were supported by the positive correlation found between the number of neutrophils infiltrating the mucosa and the degree of ICAM-1 expression on the submucosa ($p=0.0008$, $r=0.59$) and serosa ($p=0.031$, $r=0.40$), and between the degree of ICAM-1 expression and the number of infiltrating macrophages in the mucosa ($p=0.009$, $r=0.48$) and submucosa ($p=0.0013$, $r=0.57$).

Similarly to what observed for P-Selectin, ICAM-1 expression was also observed to be reduced in areas with features of necrosis, and much higher in areas with active inflammation.

ICAM-1 expression on the mucosa, submucosa, or serosa was not positively correlated to the severity of histological injury, or to clinical measures of outcome (sepsis, number of organs in failure, or survival) on multiple regression analysis.

7.3.6 E-Selectin expression

Expression of E-selectin was either absent or extremely low on all specimens from control patients and on most specimens from NECs, with only five specimens from four NEC patients showing strong staining for E-Selectin. E-Selectin expression was particularly strong on medium and large calibre vessels of the submucosa (Figure 7.23). Some E-Selectin positive blood vessels showed leukocytes adhering to the endothelium on high power magnification (Figure 7.24), although this was not as frequent as for P-Selectin and ICAM-1.

Interestingly, review of clinical data showed that such up-regulation of E-Selectin was associated with severe and rapidly evolving NEC. These four patients were all classified as Bell's stage 3 disease, required surgery one to three days after NEC was diagnosed, and presented failure of 6, 4, 4 and 3 organs respectively. When the impact of E-Selectin on clinical outcome was assessed by multiple regression analysis, E-Selectin was found to be the only factor influencing the number of organs in failure ($p=0.0005$) and survival ($p=0.0026$).

7.4 DISCUSSION

Inflammation is a dominant histological feature in intestinal specimens from patients with NEC, with up to 78% of specimens presenting microscopic signs of acute inflammation including predominance of neutrophil infiltrate (Ballance et al, 1990). The development of an inflammatory response has often been regarded as an appropriate host response to tissue necrosis and bacterial overgrowth in the intestine (Ballance et al, 1990). However, in recent years inflammatory mediators (e.g. PAF, TNF- α , IL-2, IL-6, IL-12 and IL-18) have been shown to play a central role in the development of tissue injury in experimental (Ewer et al, 2004; Hsueh et al, 1986a; Sun et al, 1988; Halpern et al, 2002) and clinical (Morecroft et al, 1994b; Caplan et al, 1990; Ng et al, 2003) NEC. Activated neutrophils and

macrophages release a wide array of proteolytic enzymes (including elastase, gelatinase and collagenase; Moraes et al, 2003) and reactive oxygen species (Cuzzocrea et al, 2001b), necessary for transepithelial migration and bacterial killing. This may result in excessive host tissue damage, so that infiltration of leukocytes in the intestine may actively contribute to the development of tissue damage in NEC. This is supported by the decreased degree of intestinal injury in a rat model of NEC where neutropenia was induced with vinblastine (Musemeche et al, 1991), or where macrophages were inhibited with semapimod (Zamora et al, 2005).

Expression of adhesion molecules on the surface of endothelial cells, in response to interleukins, TNF- α , LPS, histamine, PAF and other soluble mediators (Pettersen et al, 2002), mediates both initial rolling of leucocytes along the endothelium, and their subsequent firm adhesion followed by transendothelial migration (Ebnet et al, 1996). P-selectin, a transmembrane protein stored in Weibel-Palade bodies of endothelial cells, is responsible for the early stages of leukocyte-endothelial interaction. Within 10-30min after exposure to appropriate inflammatory stimuli, P-selectin is mobilised and exposed on the cell surface, where its interaction with PSGL-1, a specific ligand expressed by leukocytes, determines tethering and rolling of neutrophils along the endothelium (Ley et al, 1995). Sustained P-Selectin expression from de-novo synthesis occurs within a few hours from the initial stimulus (Eppihimer et al, 1997).

Expression of E-Selectin requires de-novo synthesis, and follows prompt release of P-Selectin on endothelial cell membrane, reducing rolling velocity of leukocytes, and mediating the transition from slow rolling to firm leukocyte arrest, which are mediated by different ligands on leukocyte surface (Xia et al, 2002; Smith et al, 2004; Hidalgo et al, 2007).

The role of P-Selectin and ICAM-1 in leukocyte migration through the endothelium has been extensively investigated in experimental models relevant to human NEC, such as ischaemia and reperfusion injury, whereas there are relatively few data that support a role for E-selectin in the same recruitment process. This may result both from the fact that most studies focus on the inflammatory events that occur within the first hour after reperfusion, and from the use of anti-human

monoclonal antibodies with limited binding affinity to rodent E-selectin. In fact, immunostaining for E-Selectin was attempted in the experimental studies described in Chapter 4, 5, and 6 with the same goat anti-human E-Selectin antibody successfully used for the experiments on human NEC described in this chapter, but no satisfactory staining was obtained probably due to poor cross reactivity with the rat epitopes. Blockade of neutrophil adhesion to monolayers of TNF-activated endothelial cells by an anti-murine E-selectin monoclonal antibody proved to inhibit accumulation of neutrophils in mice small intestine following mesenteric reperfusion (Russell et al, 2000). As for P-Selectin, upregulation of E-Selectin appears to be triggered by reactive oxygen species (particularly superoxide) and cytokine release (including TNF- α) via activation of the nuclear transcription factor NF-kB (Ichikawa et al, 1997; Russell et al, 2000).

Following tethering and rolling along the vessel wall, leukocytes can adhere to and subsequently migrate through the endothelium, mediated by other adhesion molecules expressed by activated endothelium (ICAM-1, VCAM-1), and their specific counterparts on leucocytes.

My results from resected bowel in neonates with advanced NEC support the hypothesis of an upregulation of endothelial adhesion molecules P-selectin and ICAM-1. Expression of P-selectin was substantially increased on both medium-sized vessels and on the microcirculation of NEC patients, and was not limited to the mucosa and submucosa but extended to the muscular and subserosal layer. Both neutrophils and macrophages were found adhering to endothelial cells expressing P-selectin, supporting the role of this adhesion molecule in promoting neutrophil rolling and firm adhesion to the endothelium.

The primary role for P-Selectin in allowing neutrophil rolling was confirmed by the strong correlation found between the degree of neutrophil infiltration in the mucosa and the intensity of expression of P-selectin on both medium-sized vessels and on the microcirculation. On the contrary, no correlation was found between expression of P-Selectin and number of infiltrating macrophages. Although P-Selectin is believed to be one of the fundamental ligands regulating trafficking of monocytes in intestinal microvasculature (Ishii et al, 2004), these findings may

reflect the importance of alternative mechanisms regulating monocyte rolling, including E- and L-Selectin (Sperandio, 2006).

Upregulation of ICAM-1 was also substantially enhanced on the endothelium of mucosal, serosal, and in particular submucosal blood vessels of NEC patients. Similarly to P-Selectin, ICAM-1 positive vessels showed numerous adhering leukocytes, including both neutrophils and macrophages, and there was a strong correlation between ICAM-1 expression and neutrophil and macrophage infiltrate, particularly for submucosal vessels.

Maximal expression of P-selectin and ICAM-1, associated with a conspicuous amount of leukocytes infiltrating the mucosa, was observed in areas characterised by active inflammation but limited injury, whereas in severely damaged tissue diffuse neutrophil and macrophage infiltration was present, but staining for P-selectin and ICAM-1 was consistently weak. This finding supports a role for leukocytes in actively contributing to the pathogenesis of intestinal damage. However, as the specimens in this study were from advanced NEC (resected bowel), we do not know at which stage during the development of the disease increased adhesion molecule expression occurs. In areas where severe histological damage has occurred, down-regulation of P-selectin and ICAM-1 surface expression may prevent further migration of leukocytes and limit the extent of inflammatory response. Whether this decreased adhesion molecule expression is a regulated process or merely a consequence of proteolysis and necrosis is unknown.

The pattern of expression of E-Selectin appeared to be different from that of ICAM-1 and P-Selectin, being present in only five specimens from four NEC patients, and virtually absent in all other NEC specimens, as well as in controls. Interestingly, patients with strong intestinal expression of E-Selectin presented with rapidly evolving and severe NEC, as indicated by the presence of Bell's stage 3 disease, presence of multiple organ dysfunction syndrome, need for early surgery, and adverse outcome. This was confirmed by the fact that E-Selectin (but not P-Selectin or ICAM-1) expression was found to be the only single factor influencing distant organ failure and survival in a multiple regression analysis model. Endothelial expression of E-Selectin may reflect a particularly intense

inflammatory response resulting in enhanced recruitment of inflammatory cells and subsequent development of local and distant organ injury. However, activated and soluble E-Selectin have recently been shown to initiate intracellular signalling, and E-Selectin upregulation may act through pathways independent from simple leukocyte infiltration. In fact, activation of E-Selectin by specific ligands has been shown to induce phosphorylation of extracellular signal-regulated kinase (ERK) in human colon cancer cells (Suzuki et al, 2007). E-selectin has also been involved in induction of apoptosis in endothelial cells induced by allergenic blood perfusion in a model of mouse isolated lung, which occurred through activation of Fas ligand (FasL) and protein tyrosine phosphatase pathways (Joucher et al, 2005). Soluble E-Selectin, which is shed from its transmembrane counterpart on endothelial cells, can activate neutrophil Src family tyrosine kinases in response to PAF, regulating permissive store-operative calcium entry (McMeekin et al, 2006).

7.5 CONCLUSIONS

The present study has shown that necrotizing enterocolitis is associated with the development of a substantial inflammatory response in the affected gut, which is characterised by great numbers of infiltrating inflammatory cells, mostly neutrophils and macrophages. These inflammatory changes were associated with the presence of substantial tissue injury, suggesting that leukocytes may actively contribute to the development of intestinal damage. Upregulation of P-Selectin on medium-sized vessels and on the microcirculation, and of ICAM-1 on blood vessels of the mucosa, submucosa, and serosa was observed in the majority of specimens from NEC patients. Increased expression of these adhesion molecules seems to play a crucial role in regulating leukocyte trafficking, with P-Selectin mostly mediating infiltration of neutrophils, and ICAM-1 regulating both neutrophil and macrophage influx. Finally, endothelial upregulation of E-Selectin occurs in a minority of patients, and seems to be associated with rapidly evolving NEC and distant organ failure, although the mechanisms underlying this effect are unclear.

Table 7.1. Demographics and clinical conditions of NEC and control patients at the time of surgery.

	NEC Patients (n=13)	Control Patients (n=7)	P value
Males (patients)	6 (46%)	2 (29%)	0.444*
Gestational age at birth (weeks)	26 [25-29]	38 [34-40]	0.002†
Birth weight (grams)	1000 [720-1140]	2660 [1857-3325]	0.004†
Age at surgery (days)	32 [11-40]	2 [1-5]	0.001†
Sepsis (patients)	8 (62%)	0 (0%)	0.015*
Organs in failure (number)	2 [1-2.5]	0 [0-0]	0.002†
Respiratory failure (patients)	12 (92%)	1 (14%)	0.001*
Cardiovascular failure (patients)	5 (38%)	0 (0%)	0.114*
Haematological failure (patients)	8 (62%)	0 (0%)	0.015*
Renal failure (patients)	1 (8%)	0 (0%)	1.0*
Hepatic failure (patients)	1 (8%)	0 (0%)	1.0*
Microvascular failure (patients)	3 (23%)	0 (0%)	0.521*

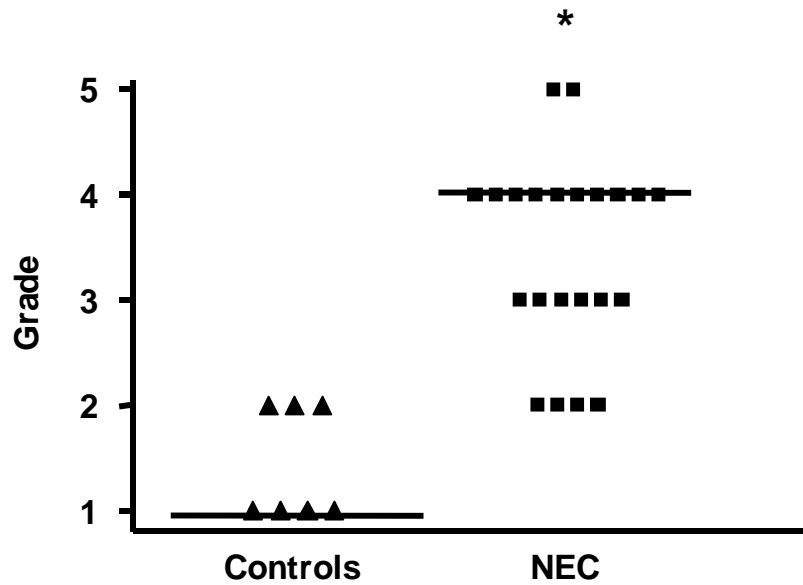
Data are expressed as median [IQ range], and were compared by Chi-Square (*) and Mann-Whitney U test (†).

Table 7.2. Full blood test values and coagulation study of NEC and control patients prior to surgery.

	NEC Patients (n=13)	Control Patients (n=7)	P value
Haemoglobin (g/100ml)	11.8 [8.9-12.7]	15.3 [13.3-16.6]	0.006
White blood cells (10 ⁹ cells/l)	5.7 [3.7-8.9]	11.2 [7.4-12.9]	0.428
Neutrophils (10 ⁹ /l)	2.5 [0.9-3.7]	4.6 [2.1-6.2]	0.634
Lymphocytes (10 ⁹ /l)	1.3 [0.9-1.9]	2.9 [2.1-6.6]	0.047
Monocytes (10 ⁹ /l)	1.0 [0.3-1.5]	0.9 [0.3-1.2]	0.812
Platelets (10 ⁹ /l)	82 [36-162]	227 [197-363]	0.003
PT (s)	17.6 [14.4-19.4]	14.3 [13.0-18.4]	0.428
a-PTT (s)	63.9 [47.6-84.2]	54.3 [46.4-62.9]	0.579
TT (s)	13.7 [13.0-14.9]	14.0 [13.6-14.2]	0.726
Fibrinogen (g/l)	2.2 [1-2.7]	1.5 [0.9-1.7]	0.074

Data are expressed as median [IQ range], and were compared by Mann-Whitney U test. PT: prothrombin time; a-PTT: activated partial thromboplastin time; TT: thrombin time.

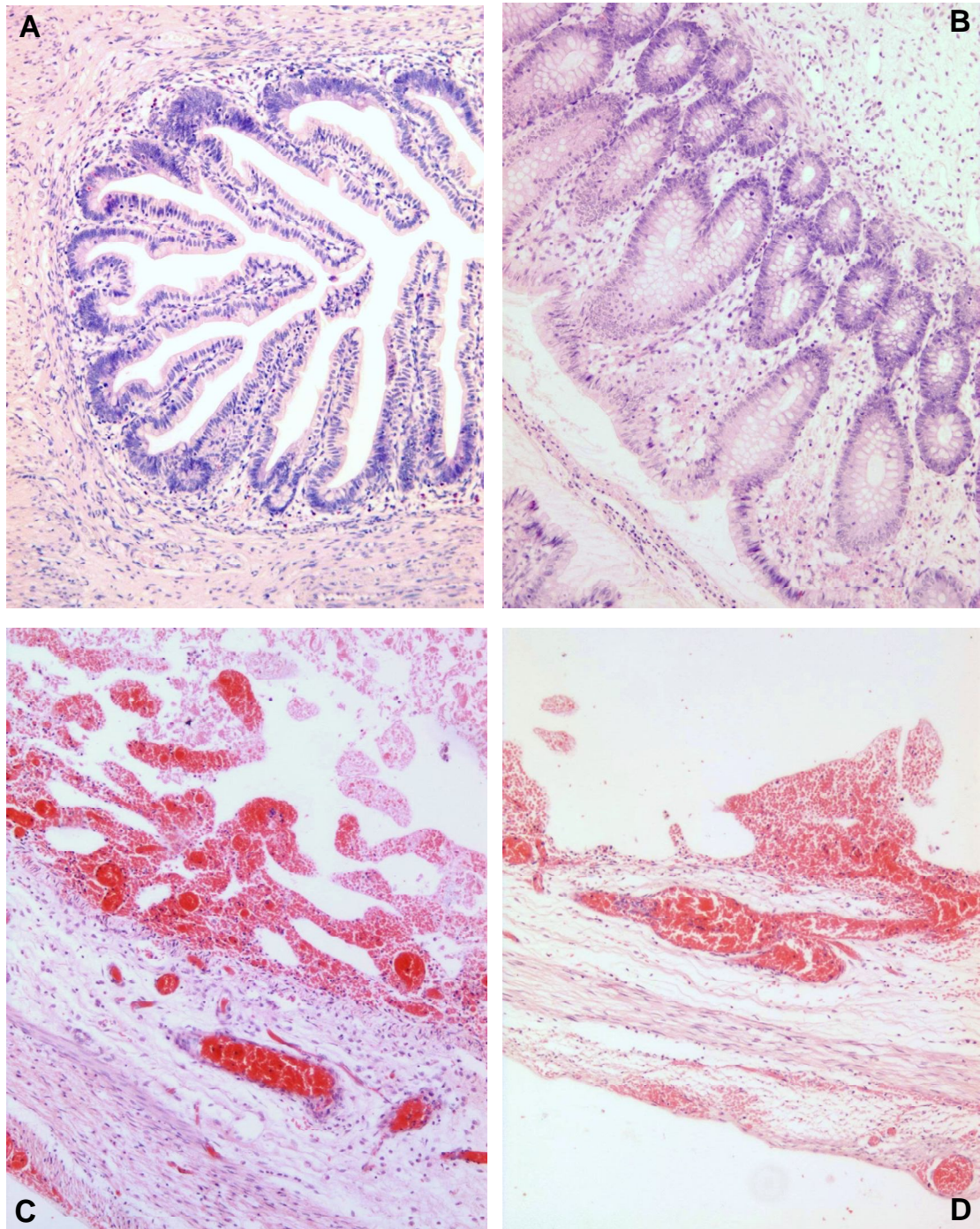
Figure 7.1. Histological injury.



Degree of histological injury in specimens from NEC patients (NEC) and control patients (Controls). Grade 1 corresponds to minimal injury; Grade 5 to maximal injury. Median is shown as a line. Results were compared using Mann-Whitney U test.

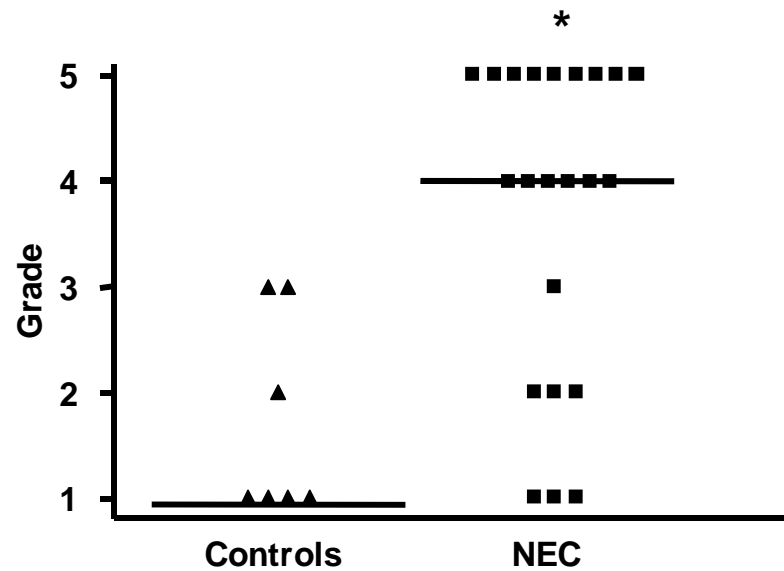
* NEC: $p=0.0003$ vs. Control.

Figure 7.2. Histological appearance.



Specimens of terminal ileum were stained with H&E and are from: Control [A]; NEC [B, C, D]. Magnification is x100.

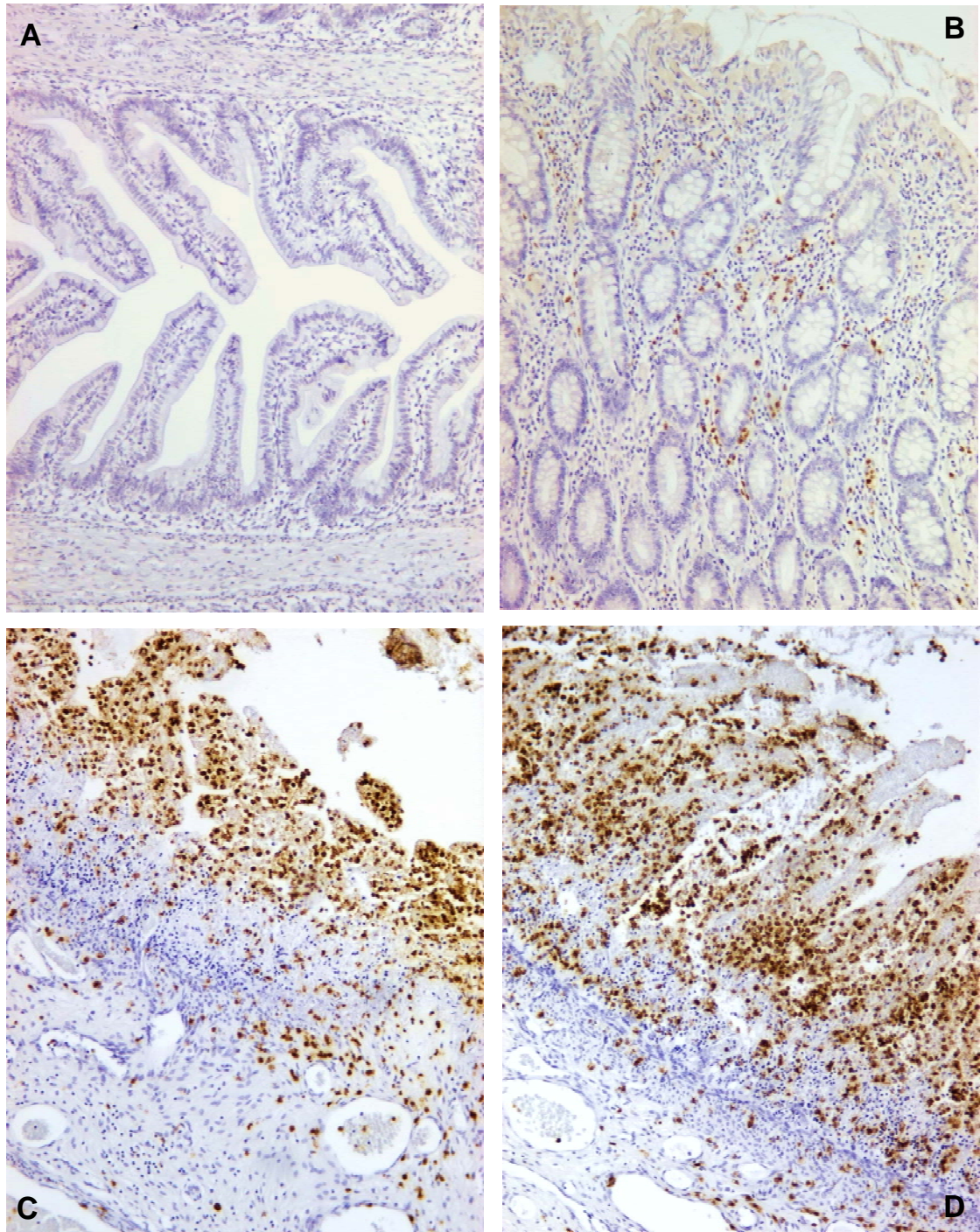
Figure 7.3. Neutrophil infiltration in the mucosa.



Degree of neutrophil infiltration in specimens from NEC patients (NEC) and control patients (Controls). Grade 1 corresponds to minimal infiltration; Grade 5 to maximal infiltration. Median is shown as a line. Results were compared using Mann-Whitney U test.

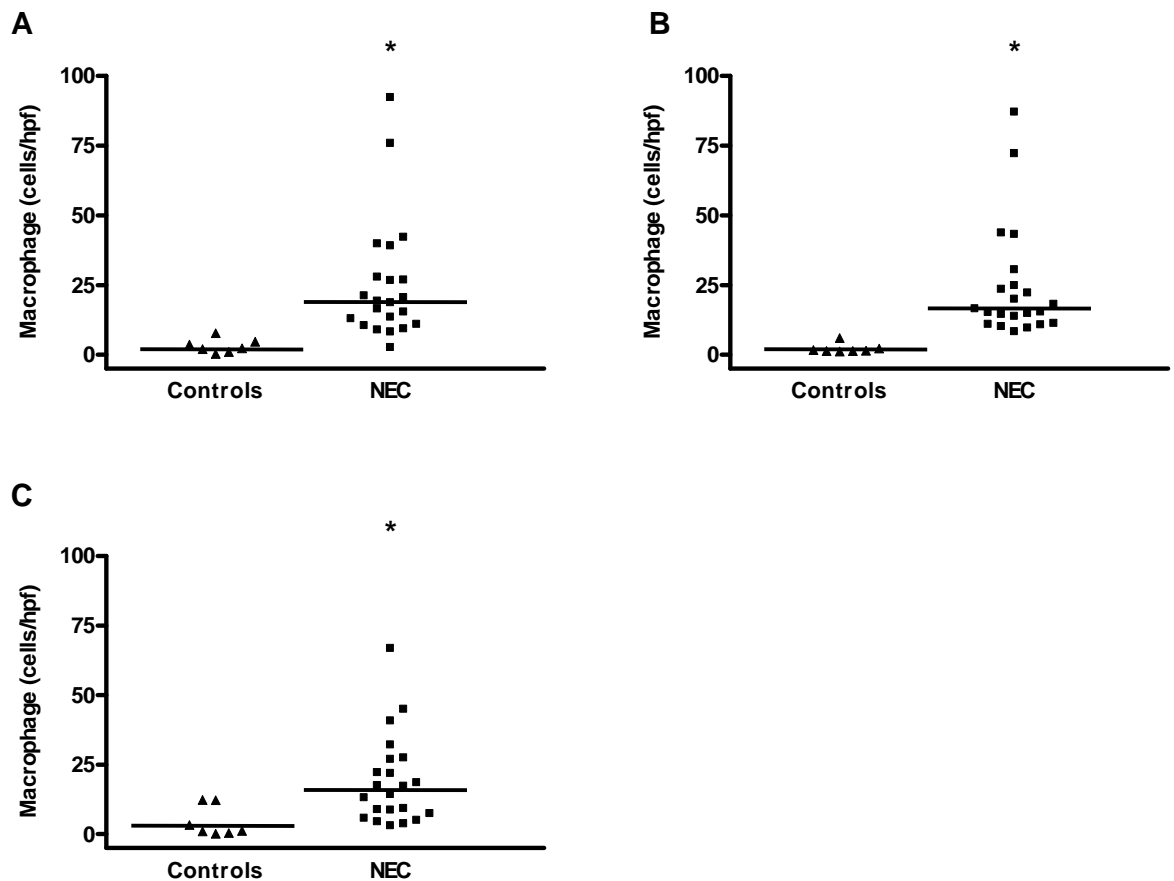
* NEC: $p=0.0068$ vs. Control.

Figure 7.4. Histological appearance of neutrophil infiltration in the mucosa.



Specimens of terminal ileum (stained with anti-neutrophil elastase antibodies and counterstained with haematoxylin) are from: Control [A, B]; NEC [C, D]. Magnification is x200.

Figure 7.5. Macrophage infiltration.



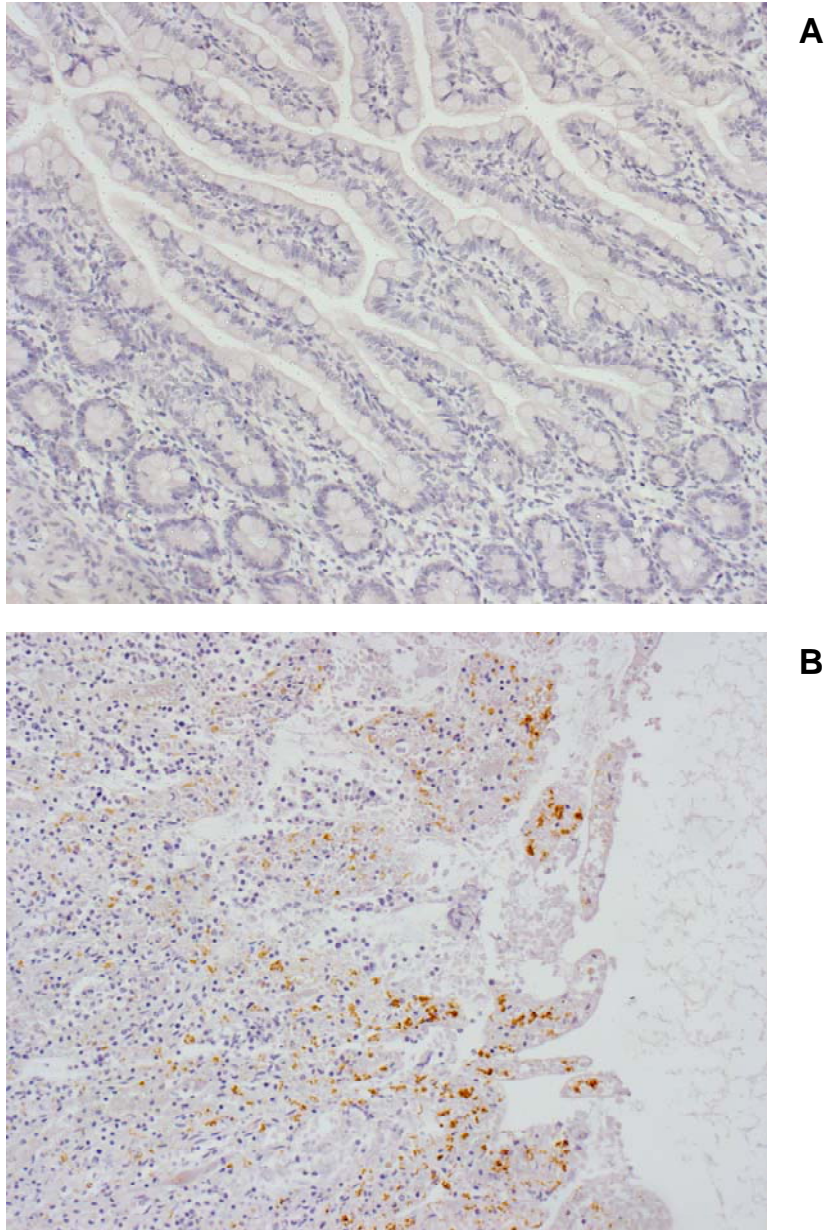
Macrophage infiltration in the mucosa [A], submucosa [B], and serosa [C] in specimens from NEC patients (NEC) and control patients (Controls). Results are expressed as average number of cells/high power field (cells/hpf) counted on 20 different fields in each specimen. Median is shown as a line. Results were compared using Mann-Whitney U test.

[A]: * NEC: $p=0.0002$ vs. Control.

[B]: * NEC: $p<0.0001$ vs. Control.

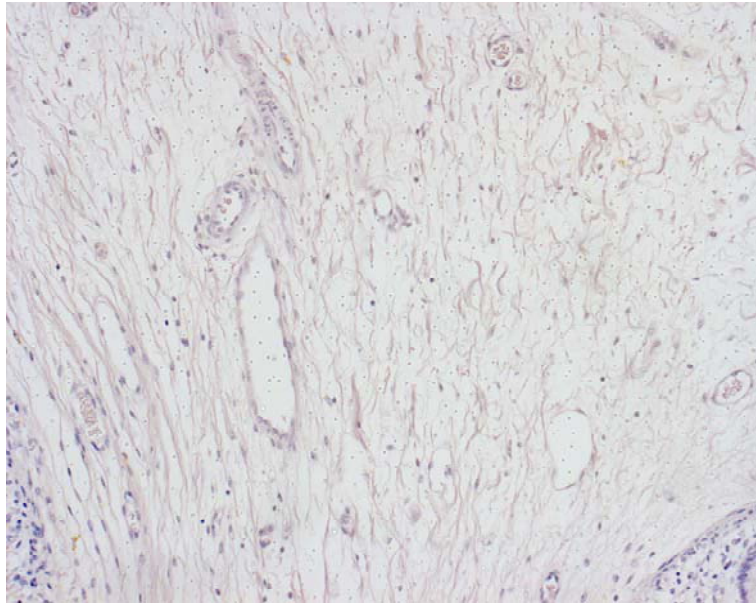
[C]: * NEC: $p=0.0034$ vs. Control.

Figure 7.6. Histological appearance of macrophage infiltration in the mucosa.

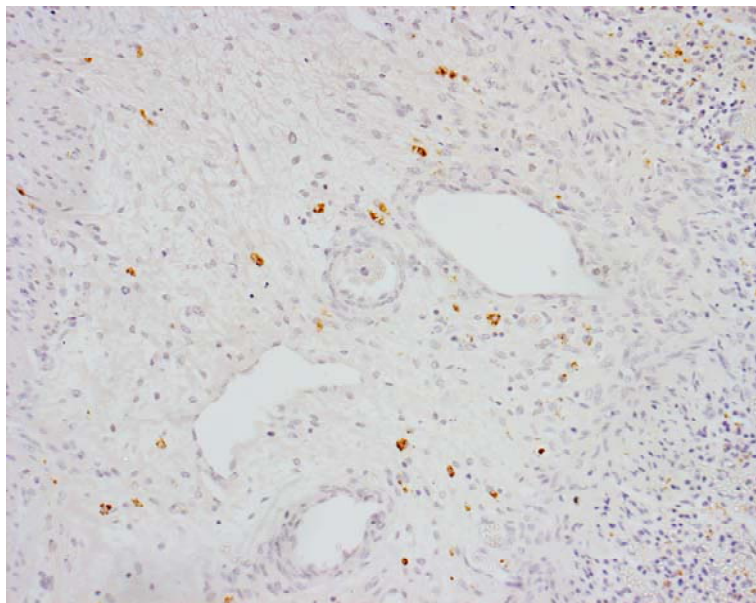


Specimens of terminal ileum (stained with anti-CD68 antibodies and counterstained with haematoxylin) are from: Control [A]; NEC [B]. Magnification is x200.

Figure 7.7. Histological appearance of macrophage infiltration in the submucosa.



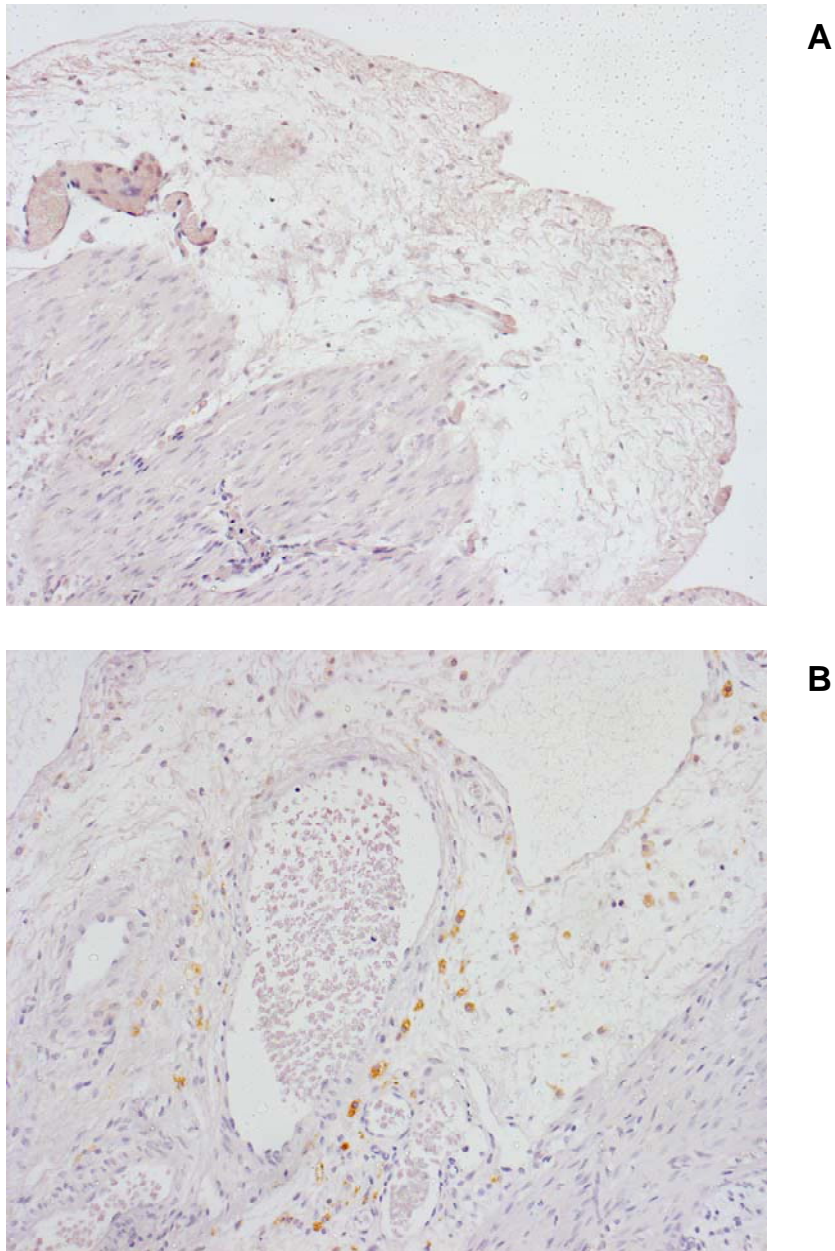
A



B

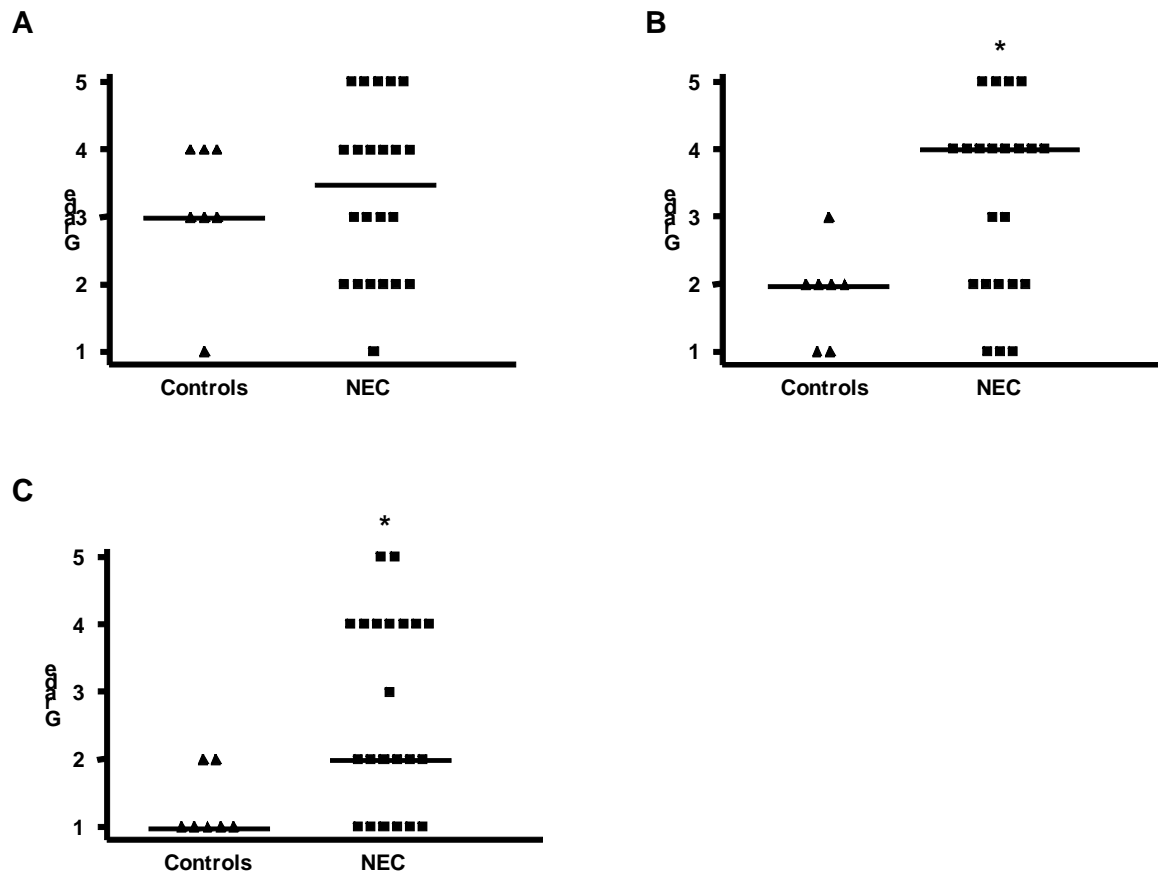
Specimens of terminal ileum (stained with anti-CD68 antibodies and counterstained with haematoxylin) are from: Control [A]; NEC [B]. Magnification is x200.

Figure 7.8. Histological appearance of macrophage infiltration in the serosa.



Specimens of terminal ileum (stained with anti-CD68 antibodies and counterstained with haematoxylin) are from: Control [A]; NEC [B]. Magnification is x200.

Figure 7.9. P-Selectin expression.

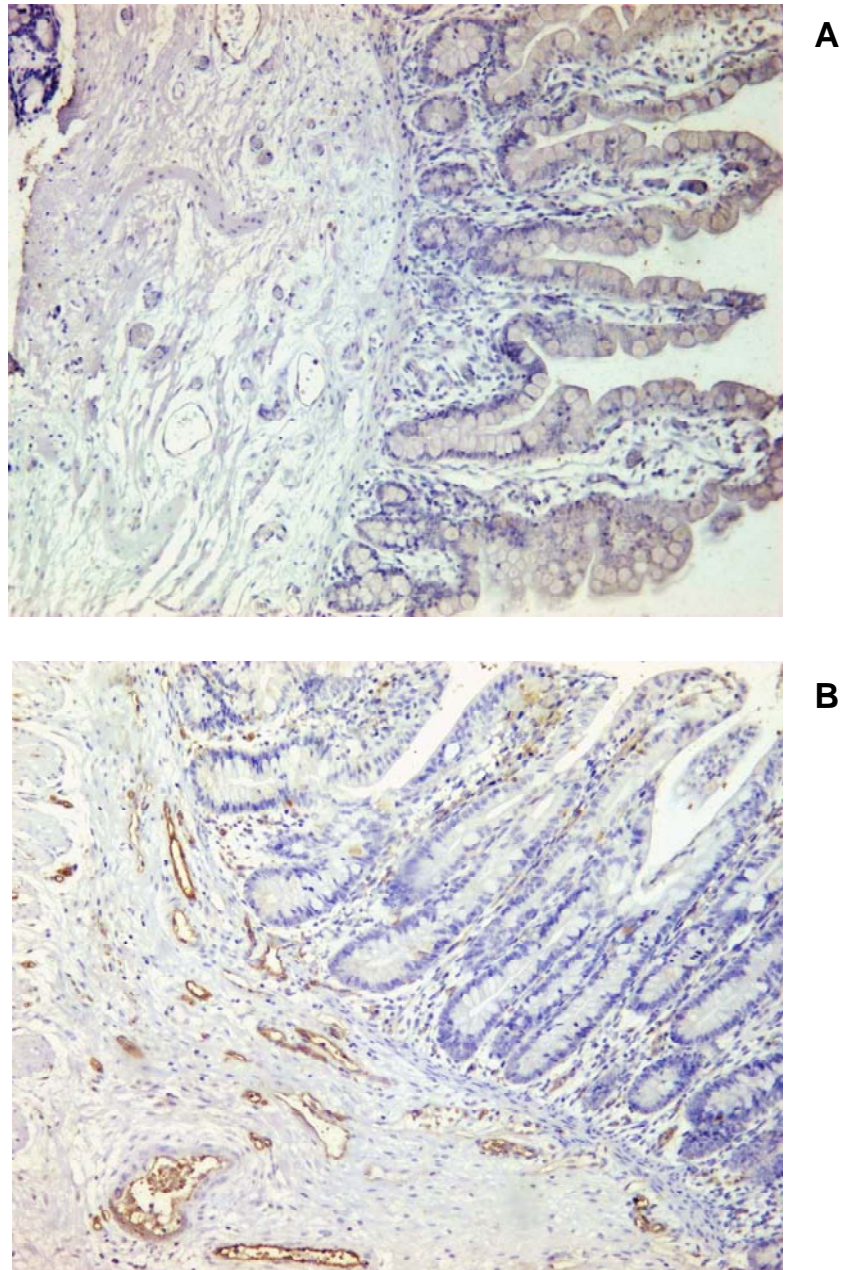


P-Selectin expression on large vessels [A], medium-sized vessels [B], and microcirculation [C] in specimens from NEC patients (NEC) and control patients (Controls). Grade 1 corresponds to minimal expression; Grade 5 to maximal expression. Median is shown as a line. Results were compared using Mann-Whitney U test.

[B]: * NEC: $p=0.0298$ vs. Control.

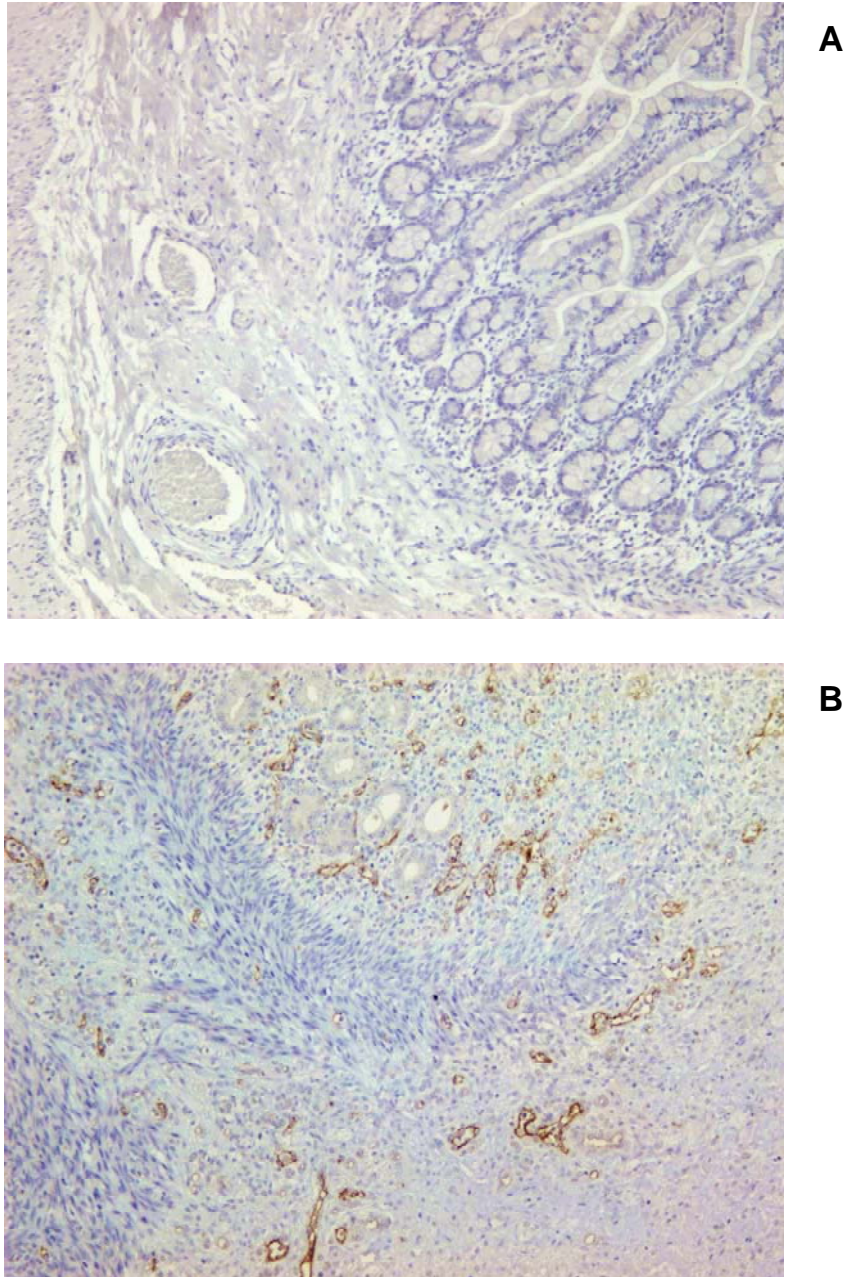
[C]: * NEC: $p=0.0259$ vs. Control.

Figure 7.10. Histological appearance of P-Selectin expression on large vessels.



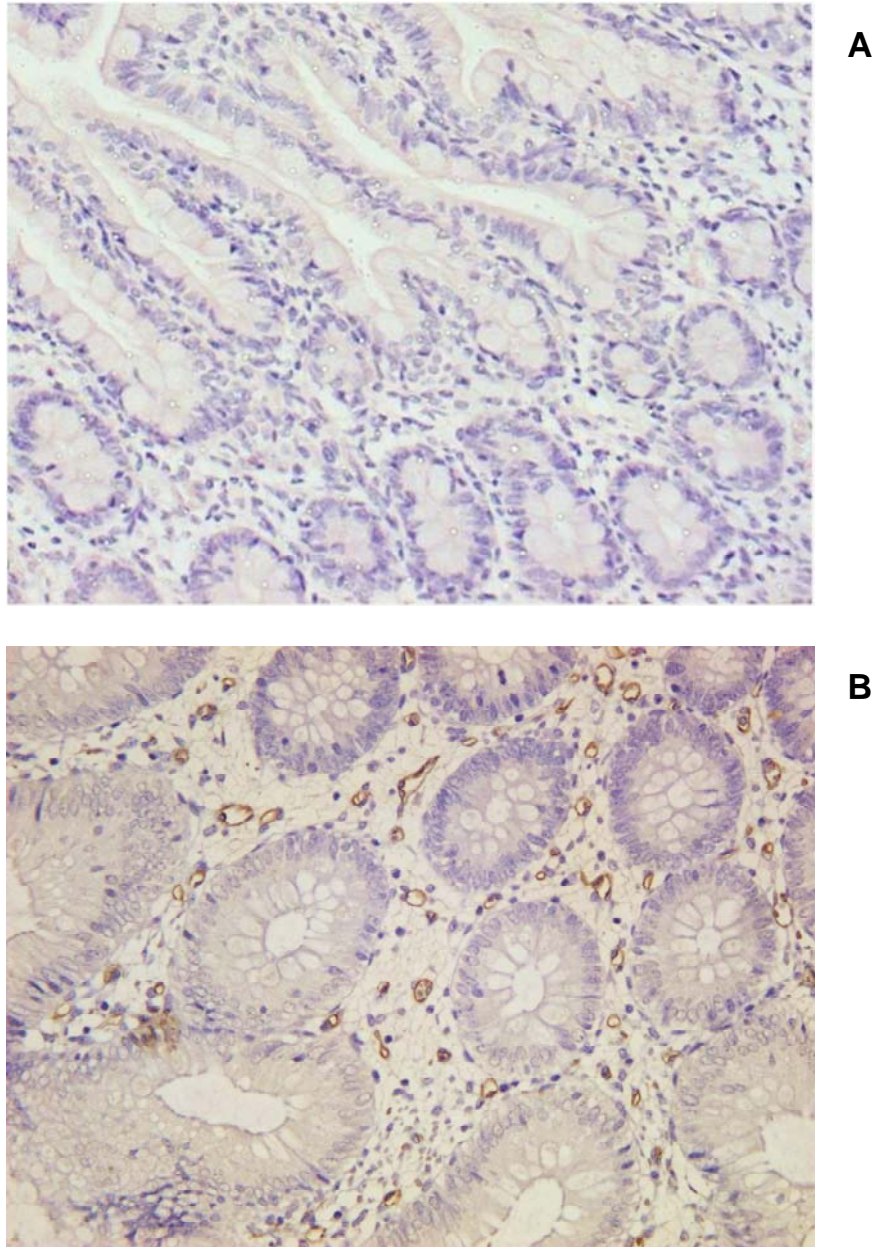
Expression of P-Selectin on large vessels in intestinal specimens from Control [A] and NEC [B] patients. Sections were stained for P-Selectin and counterstained with haematoxylin. Magnification is x100.

Figure 7.11. Histological appearance of P-Selectin expression on medium-sized vessels.



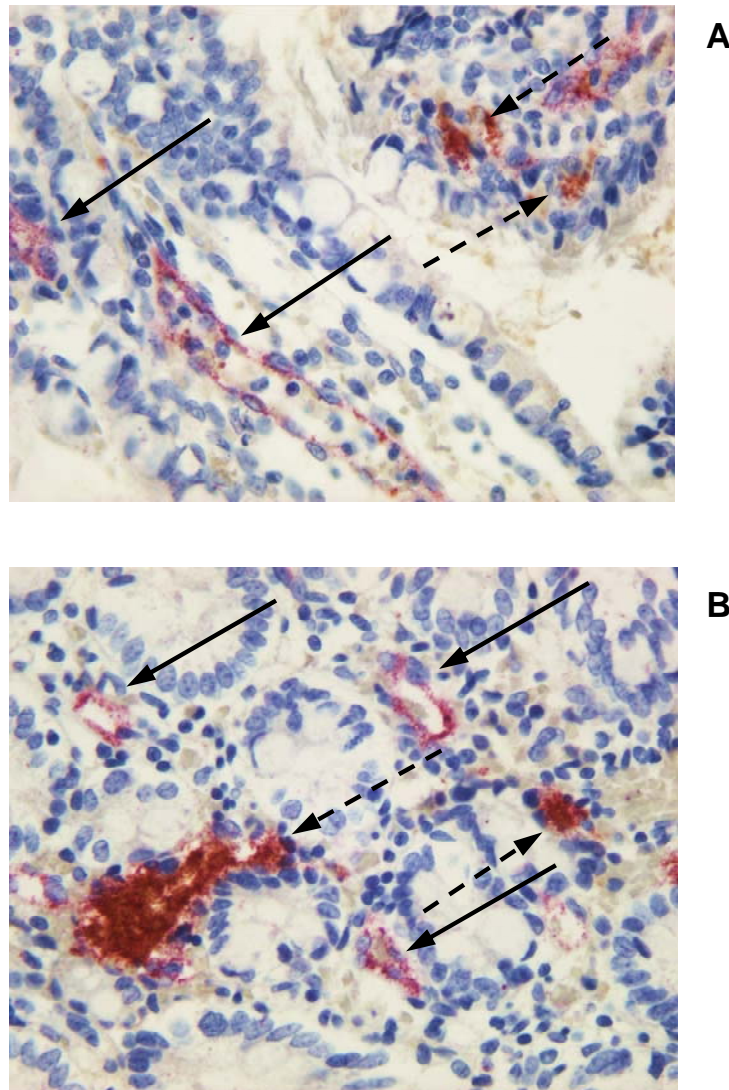
Expression of P-Selectin on medium-sized vessels in intestinal specimens from Control [A] and NEC [B] patients. Sections were stained for P-Selectin and counterstained with haematoxylin. Magnification is x100.

Figure 7.12. Histological appearance of P-Selectin expression in the microcirculation.



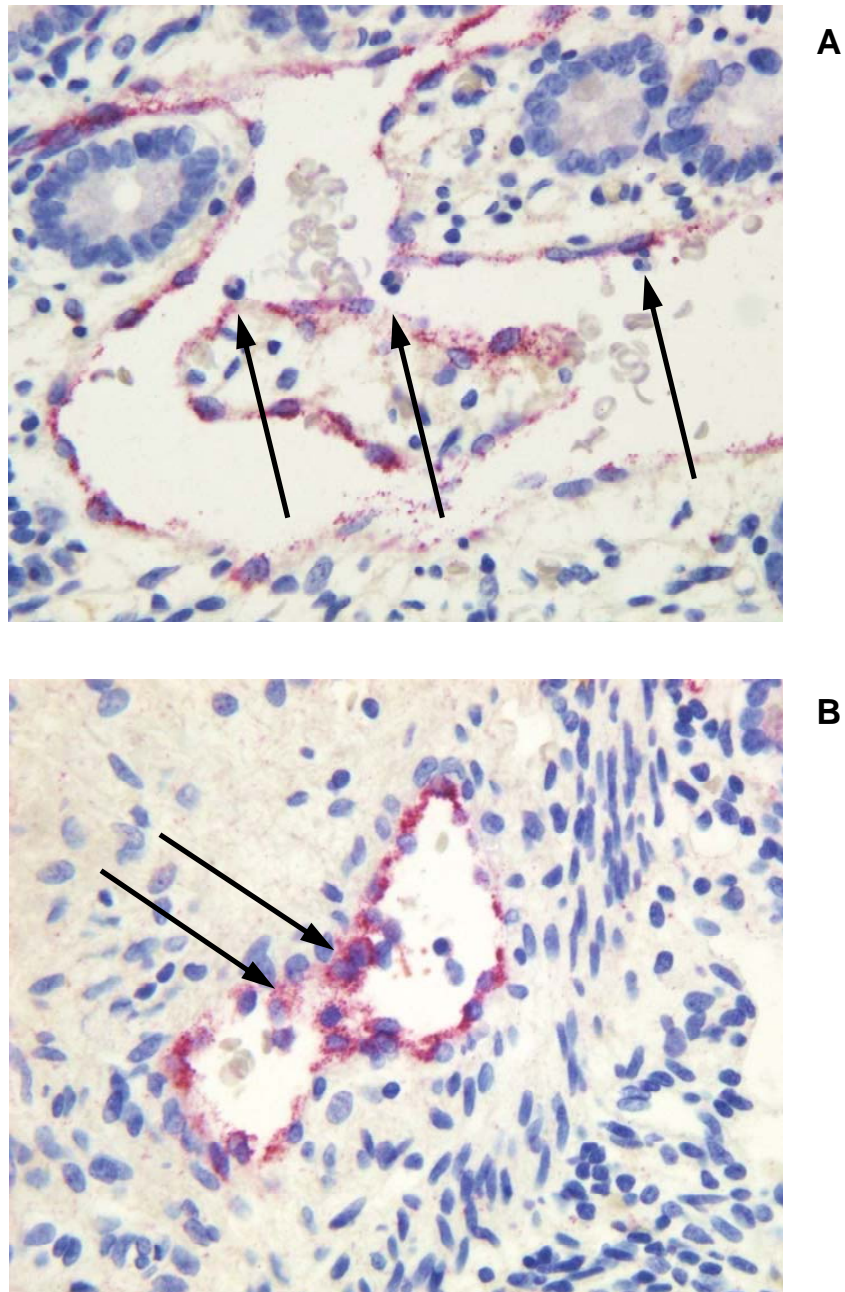
Expression of P-Selectin on the microcirculation in intestinal specimens from Control [A] and NEC [B] patients. Sections were stained for P-Selectin and counterstained with haematoxylin. Magnification is x400.

Figure 7.13. P-selectin and CD41 expression in NEC patients.



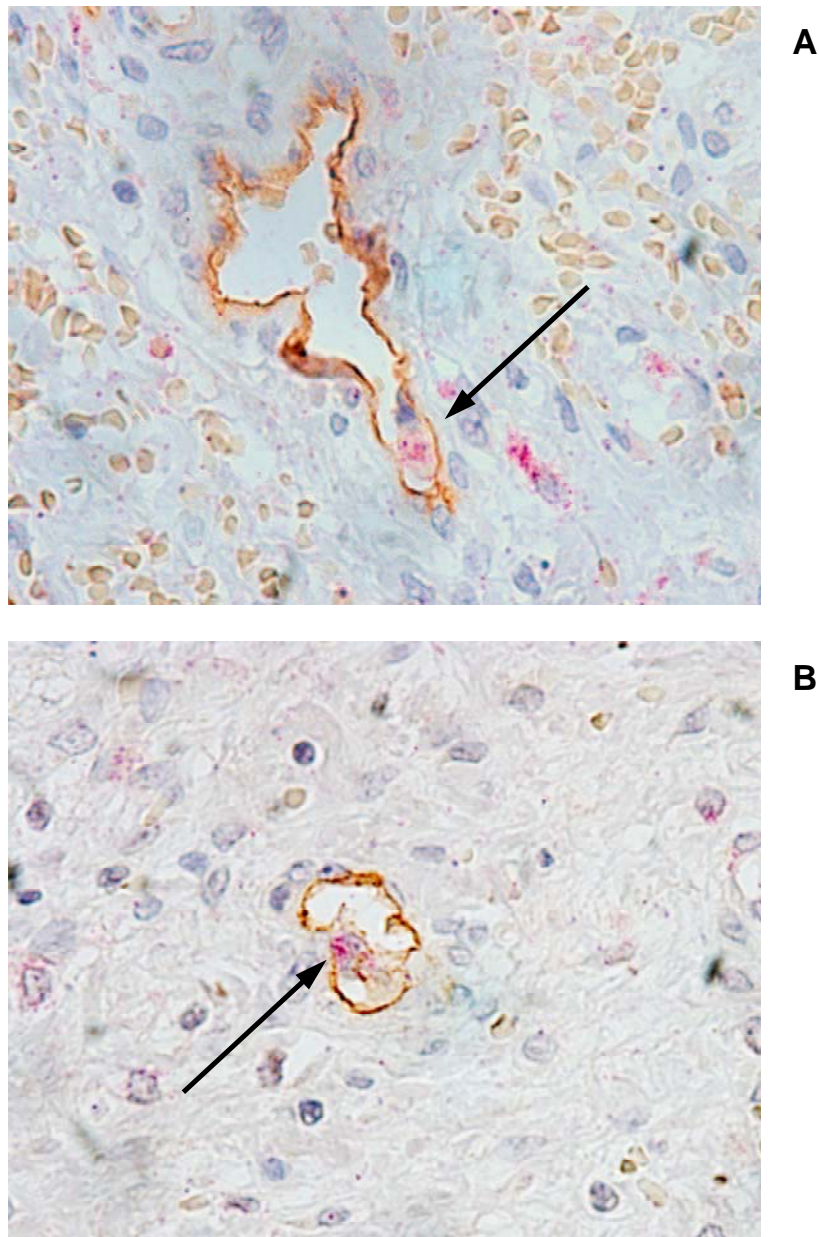
P-Selectin (fast red) and CD41 (DAB) expression on dual stained sections in specimens from NEC patients. Solid lines indicate selective expression of P-selectin on endothelial cells resulting in pink-coloured, ring-shaped appearance of blood vessels. Dashed lines indicate co-expression of P-selectin and CD41 on activated platelets, resulting in a brown-orange staining within blood vessels. Magnification is x1,000.

Figure 7.14. P-Selectin expression and leukocyte adherence.



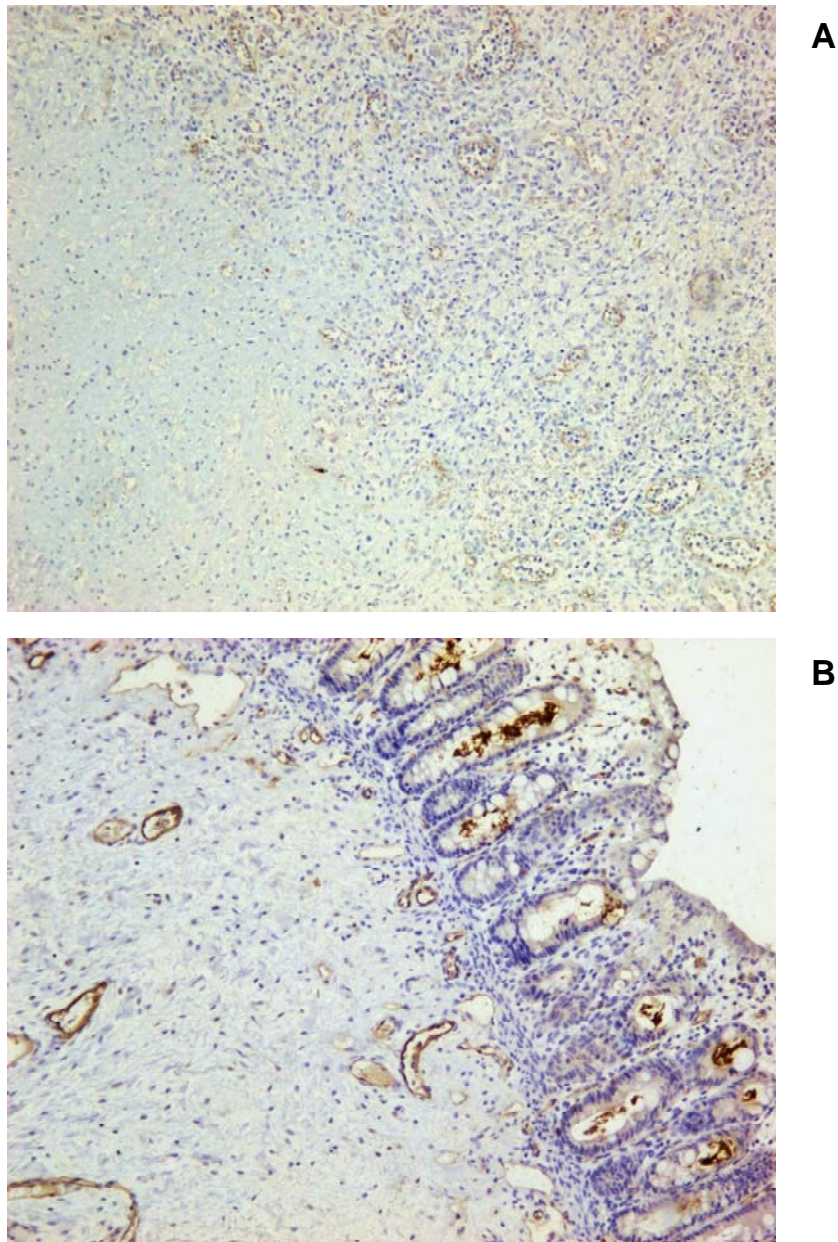
Specimens from NEC patients stained for P-Selectin (fast red) and counterstained with haematoxylin. Arrows indicate leukocytes adhering to P-Selectin positive vessels. Magnification is x1,000.

Figure 7.15. P-Selectin expression and macrophage adherence.



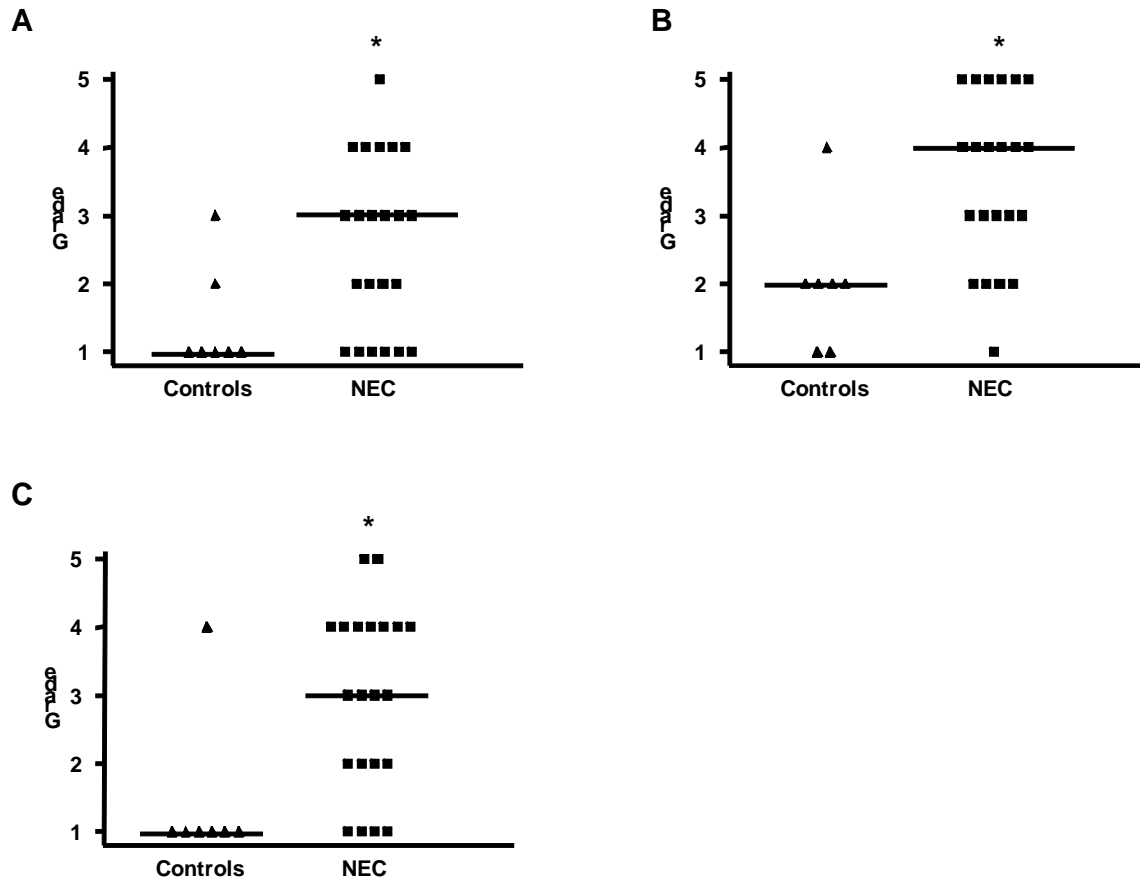
Specimens from NEC patients dual stained for P-Selectin (DAB) and CD68 (fast red), and counterstained with haematoxylin. Macrophages adhering to P-Selectin positive vessels are indicated by arrows. Magnification is x1,000.

Figure 7.16. P-Selectin expression in areas with severe and mild injury.



Sections from a single NEC patient were stained for P-Selectin and counterstained with haematoxylin. [A]: mild (large and medium-sized vessels) and weak (microcirculation) staining in an area with very severe injury. [B]: very strong (large vessels), and strong (medium-sized vessels and microcirculation) staining in an area with mild injury. Magnification is x100.

Figure 7.17. ICAM-1 expression.



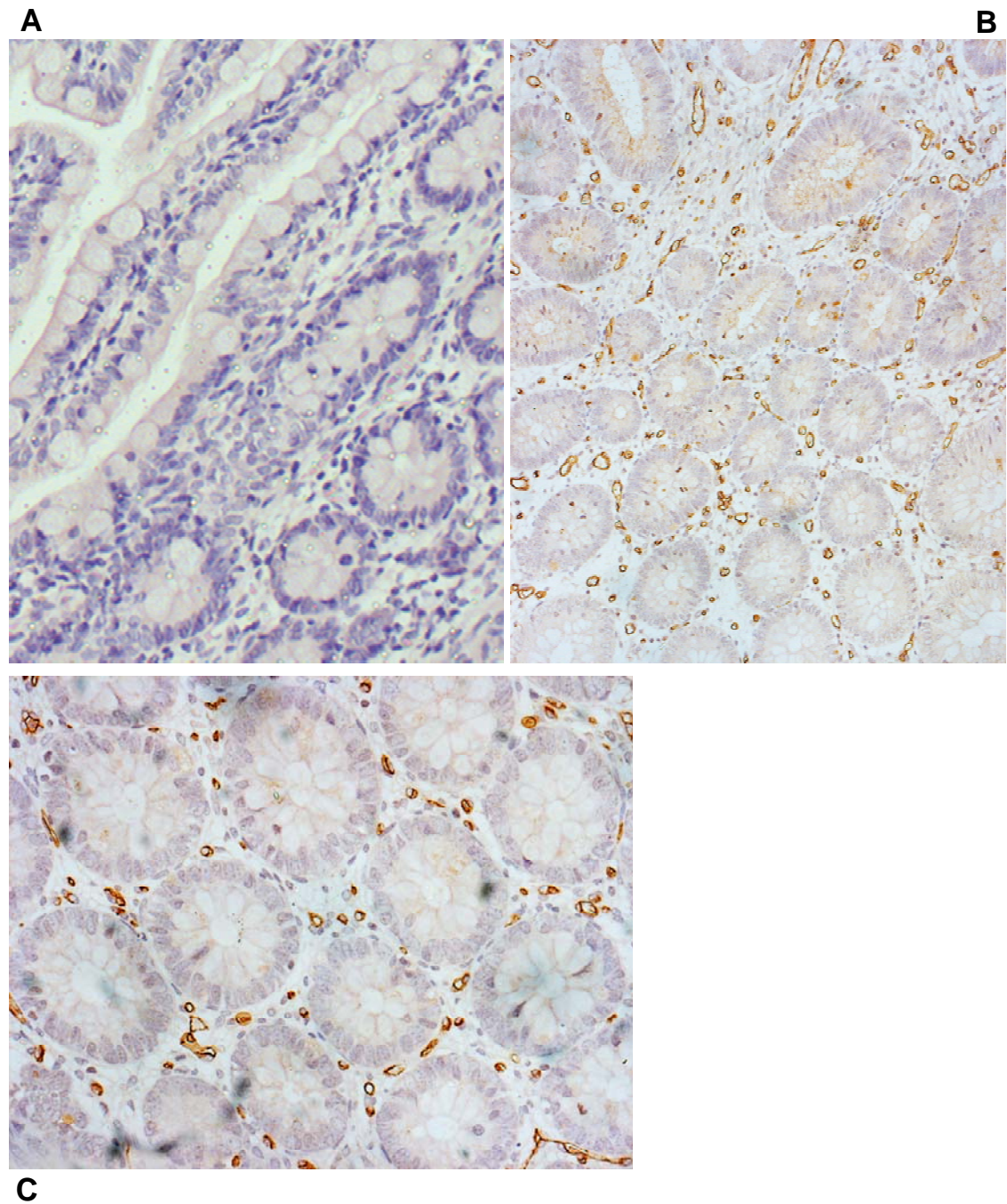
ICAM-1 expression in the mucosa [A], submucosa [B], and serosa [C] in specimens from NEC patients (NEC) and control patients (Controls). Grade 1 corresponds to minimal expression; Grade 5 to maximal expression. Median is shown as a line. Results were compared using Mann-Whitney U test. Results were compared using Mann-Whitney U test.

[A]: * NEC: $p=0.0381$ vs. Control.

[B]: * NEC: $p=0.0099$ vs. Control.

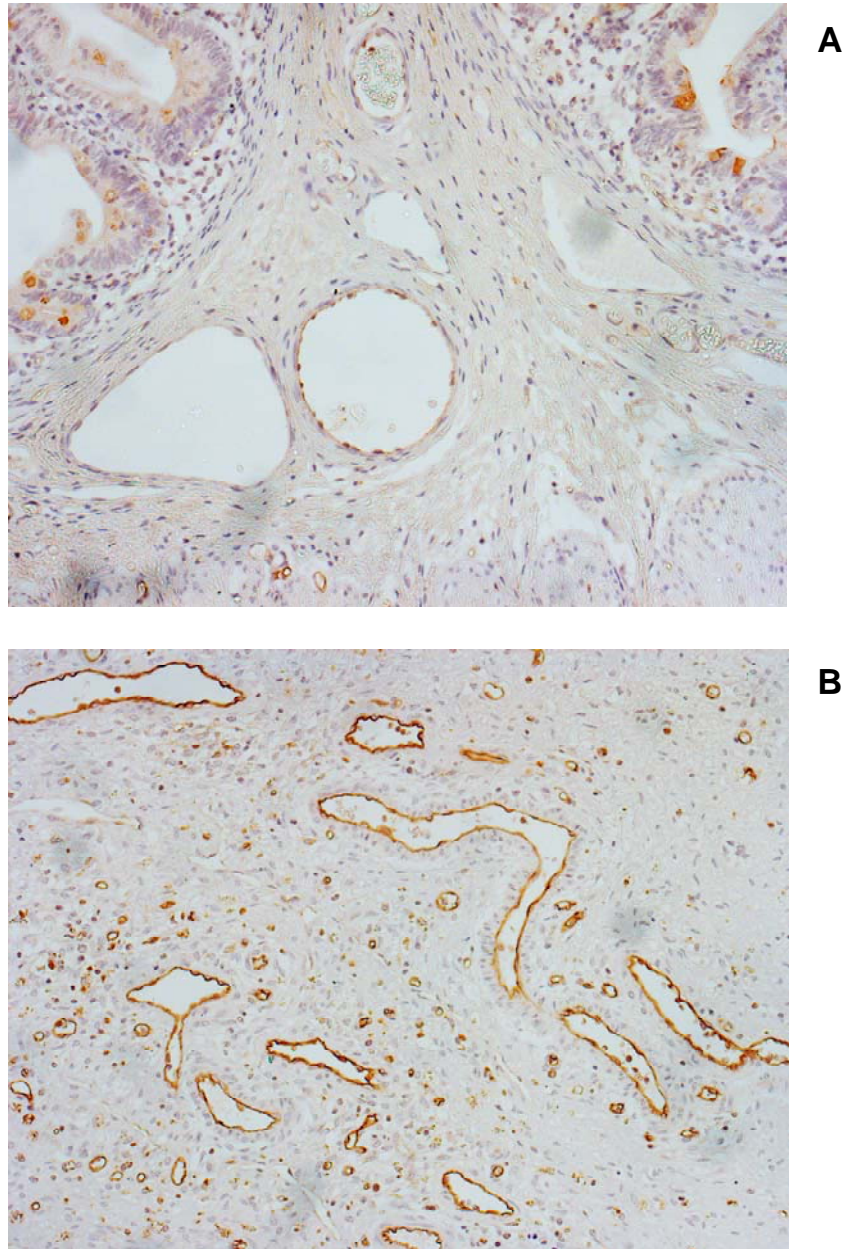
[C]: * NEC: $p=0.0153$ vs. Control.

Figure 7.18. Histological appearance of ICAM-1 expression in the mucosa.



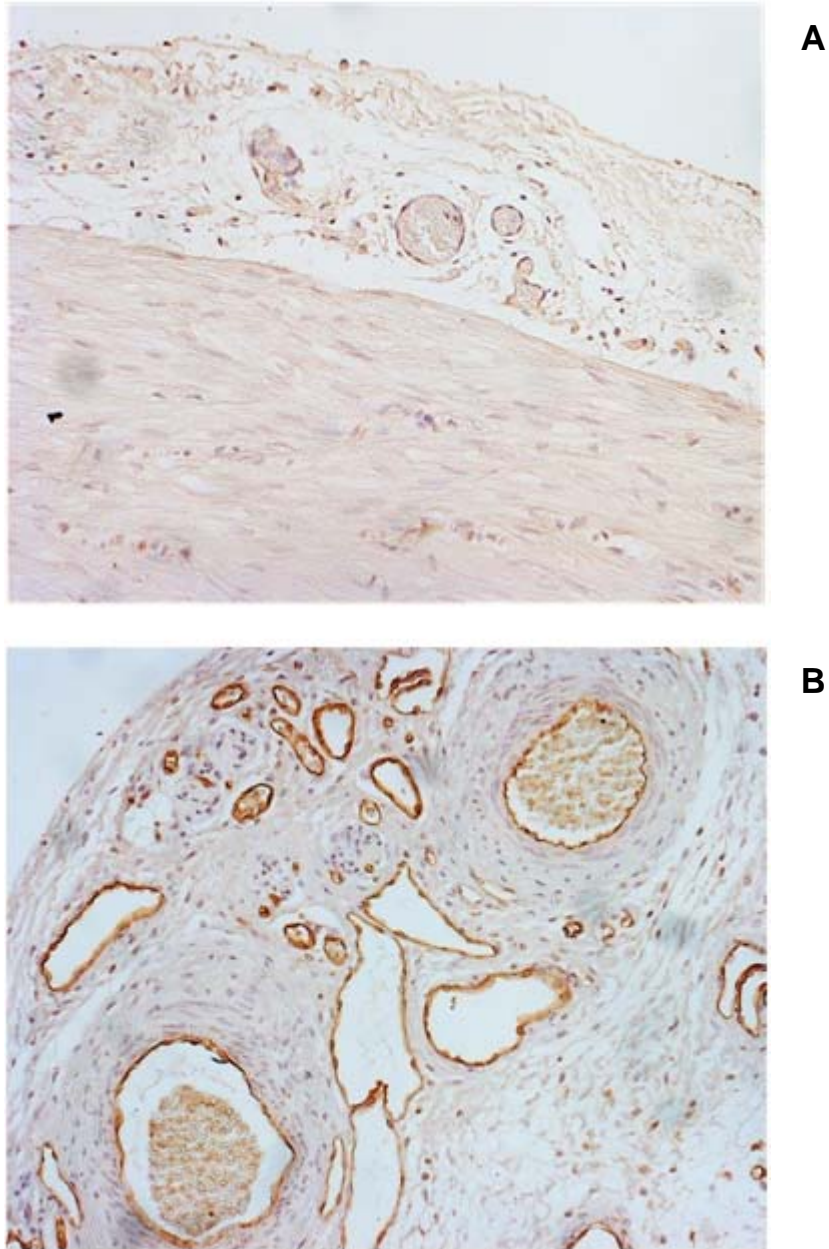
Expression of ICAM-1 on the microcirculation in intestinal specimens from Control [A] and NEC [B, C] patients. Sections were stained for ICAM-1 and counterstained with haematoxylin. Magnification is x200 [B] and x400 [A, C].

Figure 7.19. Histological appearance of ICAM-1 expression in the submucosa.



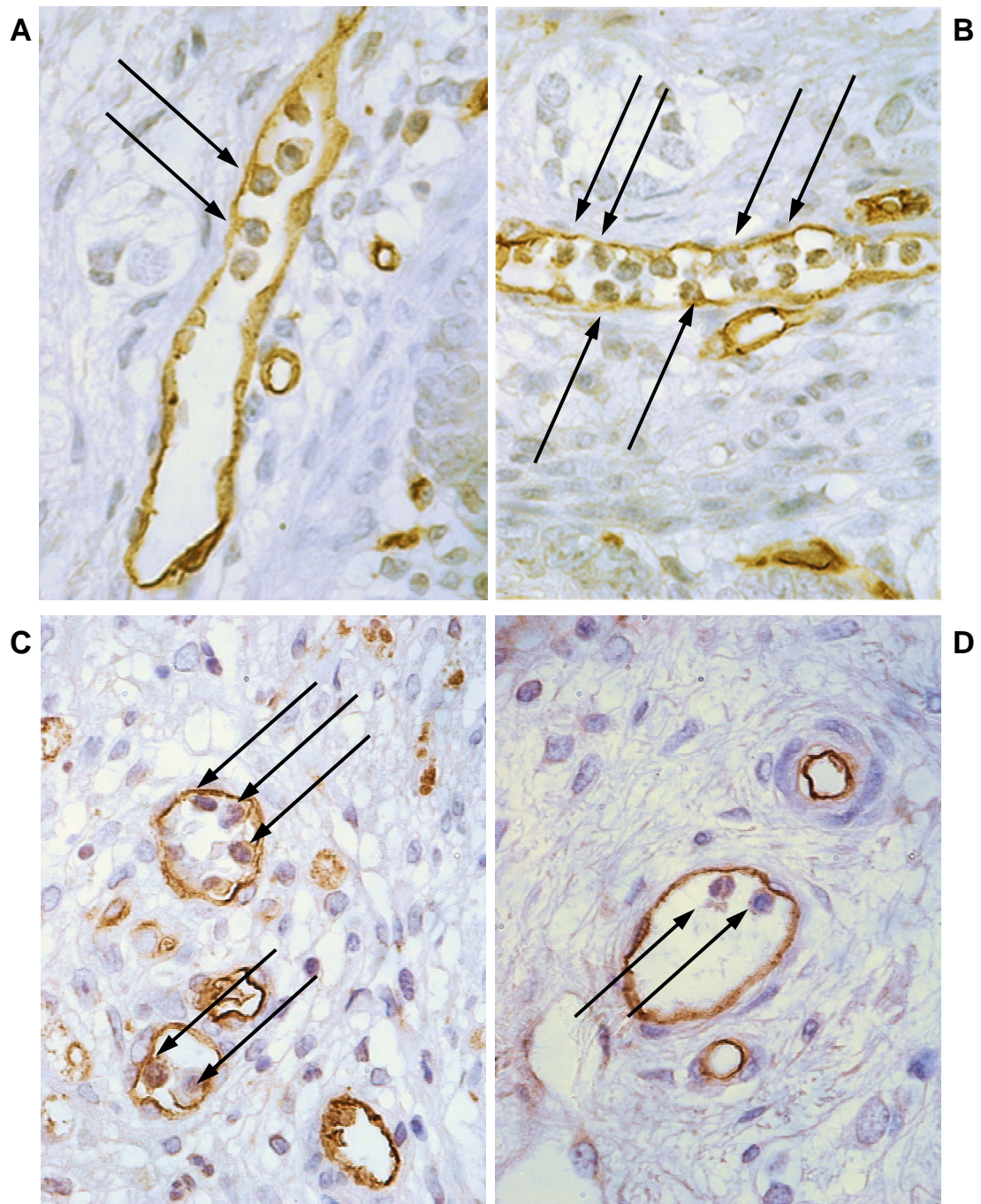
Expression of ICAM-1 on the submucosa in intestinal specimens from Control [A] and NEC [B] patients. Sections were stained for ICAM-1 and counterstained with haematoxylin. Magnification is x200.

Figure 7.20. Histological appearance of ICAM-1 expression in the serosa.



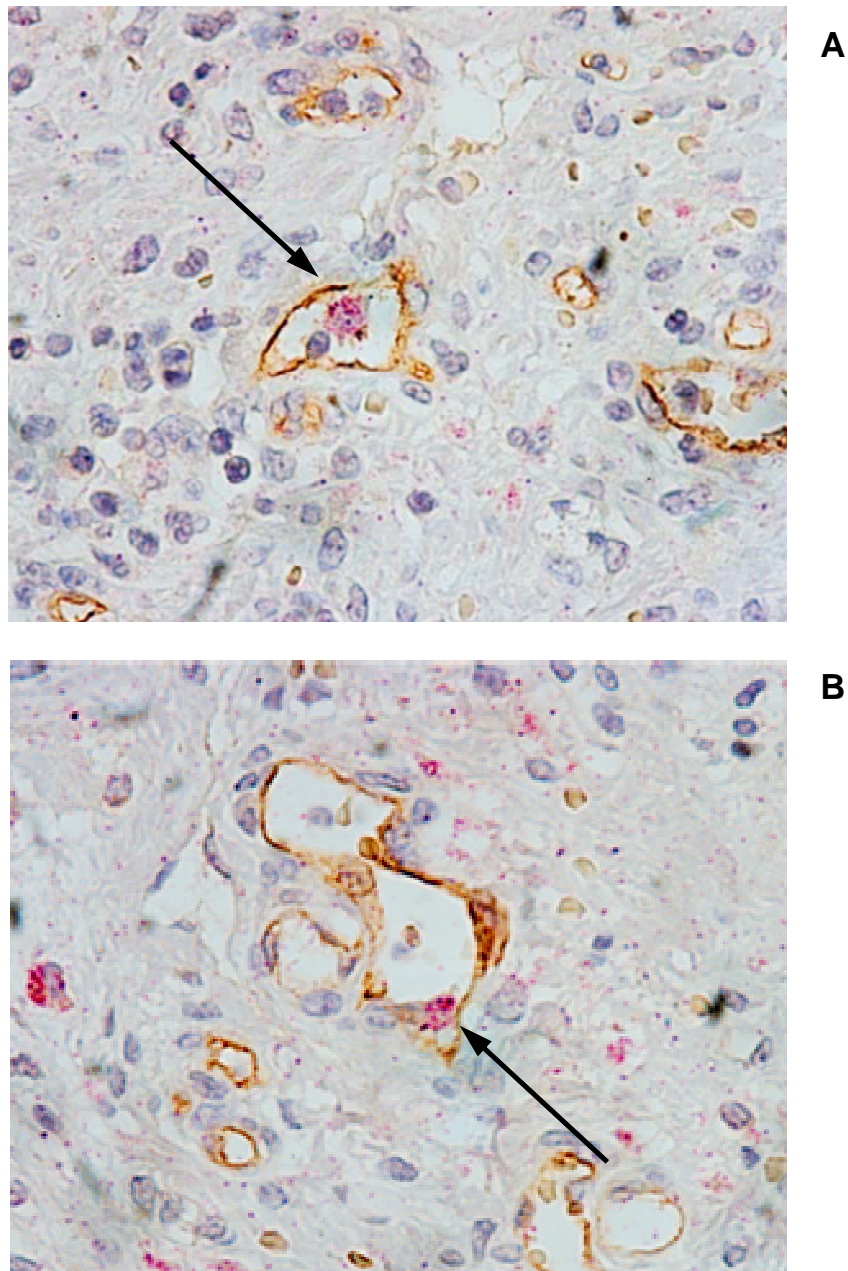
Expression of ICAM-1 on the serosa in intestinal specimens from Control [A] and NEC [B] patients. Sections were stained for ICAM-1 and counterstained with haematoxylin. Magnification is x200.

Figure 7.21. ICAM-1 expression and leukocyte adherence.



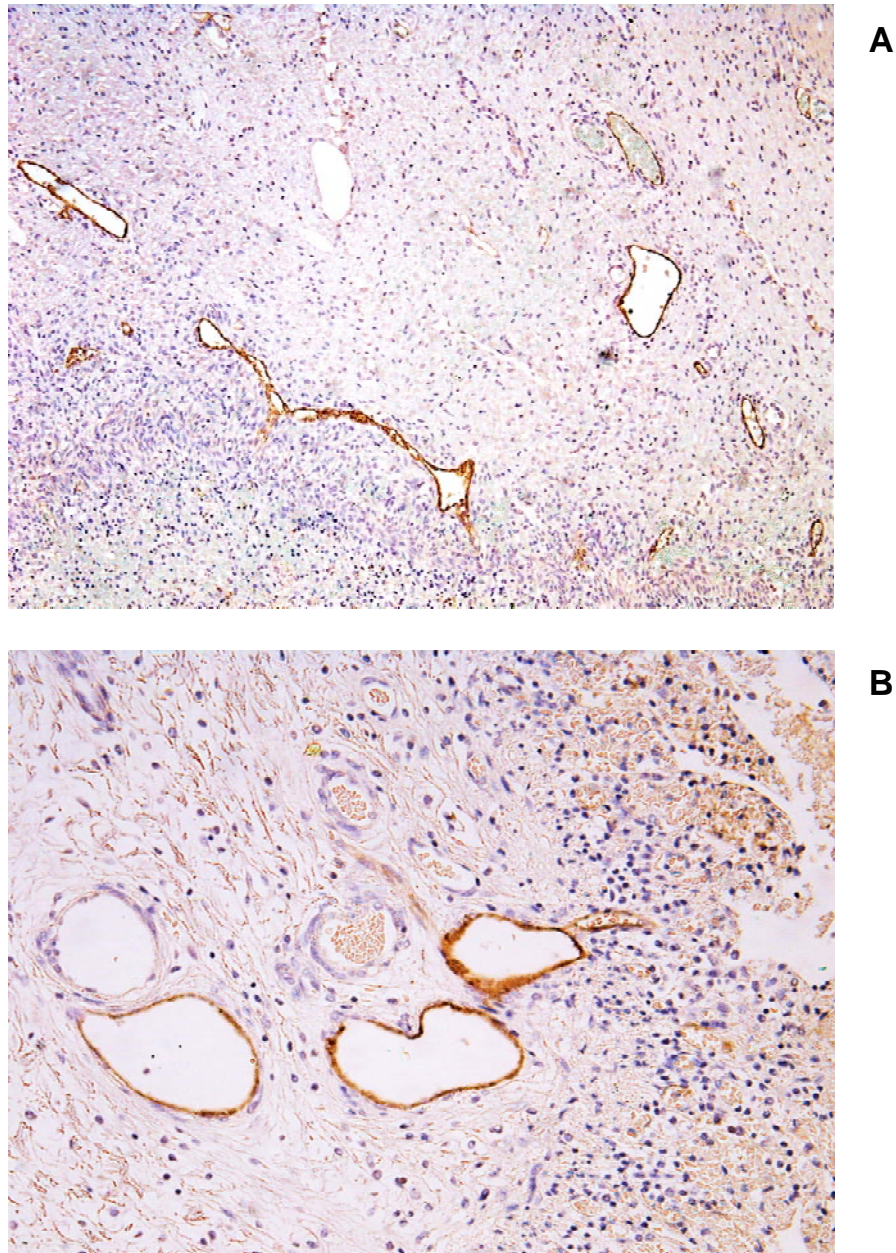
Specimens from NEC patients stained for ICAM-1 and counterstained with haematoxylin. Leukocytes adhering to ICAM-1 positive vessels are indicated by arrows. Magnification is x1,000.

Figure 7.22. ICAM-1 expression and macrophage adherence.



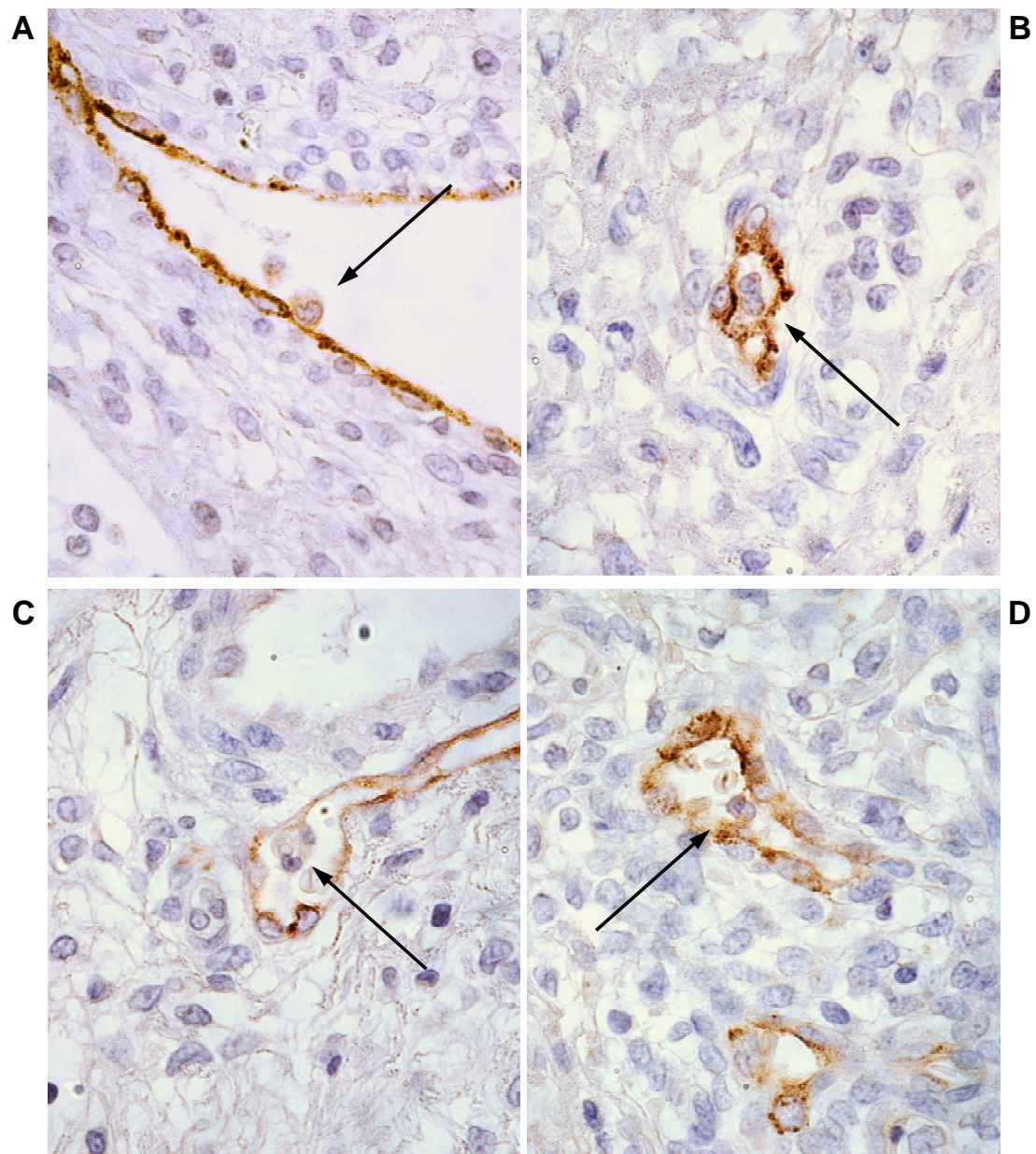
Specimens from NEC patients dual stained for ICAM-1 (DAB) and CD68 (fast red), and counterstained with haematoxylin. Macrophages adhering to ICAM-1 positive vessels are indicated by arrows. Magnification is x1,000.

Figure 7.23. E-Selectin expression in specimens from NEC patients.



Specimens from NEC patients stained for E-Selectin and counterstained with haematoxylin. Magnification is: x100 [A] and x200 [B].

Figure 7.24. E-Selectin expression and leukocyte adherence.



Specimens from NEC patients stained for E-Selectin and counterstained with haematoxylin. Leukocytes adhering to E-Selectin positive vessels are indicated by arrows. Magnification is x1,000.

CHAPTER 8

CONCLUSIONS AND FUTURE WORK

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8.1 CONCLUSIONS

My experiments have shown that both moderate hypothermia and the peroxynitrite decomposition catalyst FeTMPyP exert beneficial effects when applied as therapeutic strategies for experimental intestinal ischaemia and reperfusion. Investigation of the inflammatory response in human NEC has contributed to characterise the role of inflammatory cells and endothelial adhesion molecules in determining tissue injury and influencing clinical outcome. In addition, insight into the inflammatory response in newborns with NEC has revealed the similarities between our experimental model and human disease, suggesting that moderate hypothermia and FeTMPyP could represent novel therapies for this condition.

In the first set of experiments (see Chapter 3), I have characterised the effects of intestinal ischaemia and reperfusion on oxidative and nitrosative stress in adult rats. My results show that reperfusion-induced oxidative stress is particularly profound in the intestine, lungs, and heart, whereas kidneys and liver are relatively unaffected. The severity of oxidative and nitrosative stress at a systemic level is confirmed by the increase in plasma malondialdehyde and nitric oxide. Application of whole body moderate hypothermia before the onset of mesenteric ischaemia effectively prevented the changes induced by reperfusion. Evidence from this study suggests that this hypothermic protection is due to modulation of several different pathways in the affected organs, including a reduction in the release of reactive oxygen species, enhancement of endogenous anti-inflammatory defences, and a decrease in systemic nitrosative stress. These findings may explain some aspects of the pathophysiology underlying the beneficial effects of hypothermia on animal survival and organ injury previously demonstrated in a similar model (Kalia et al, 2002b; Vejchapipat et al, 2001).

The results from this first set of experiments support the potential application of whole body hypothermia in reducing intestinal reperfusion injury when the

ischaemic injury can be foreseen and hypothermia can be applied from the beginning of mesenteric ischaemia. In the clinical scenario, such situations include intestinal transplantation and cardiovascular procedures associated with aortic clamping. However, in the majority of clinical conditions characterised by mesenteric reperfusion injury, treatment can be applied only after the ischaemic injury has been established. This factor appears to be critical, since no studies investigating the potential effects of hypothermia when applied as a rescue therapy after the establishment of intestinal ischaemia have been published to date. In fact, it could be argued that some benefits of hypothermia exerted during the ischaemic phase (such as a reduction in basal metabolism) might be lost when cooling is applied as a rescue at reperfusion. In addition, the consequences of rewarming after a period of whole-body hypothermia should also be addressed before any clinical application can be considered: not only the protection offered by hypothermia might be lost after restoration of normothermia, but the process of rewarming could also be associated with severe adverse cardiovascular effects (Thoresen et al, 2000), especially if too rapid.

I therefore designed the first experimental study to investigate the use of whole body hypothermia when applied as a rescue therapy after the onset of mesenteric reperfusion (see Chapter 5). The animal model used in this series of experiments was modified to resemble an intensive care unit setting, including invasive monitoring of vital functions, aggressive fluid resuscitation, tracheostomy and mechanical ventilation, in order to reflect the severity of conditions associated with mesenteric reperfusion in humans. This was also necessary because the combination of reperfusion and hypothermia caused respiratory depression and death in non-ventilated animals. The results from this experiment show that even when applied at the onset of reperfusion, hypothermia prevents cardiovascular failure and abolishes mortality, and that these effects are maintained after rewarming. This is similar to findings of Kalia et al. in a rat model of mesenteric reperfusion where hypothermia was applied as a preventative treatment throughout both the ischaemic and the reperfusion phase (Kalia et al, 2002b), suggesting that a considerable part of hypothermic protection occurs during the reperfusion phase rather than during ischaemia. In fact, investigation of the early reperfusion phase in

my study revealed that even when applied as a rescue, hypothermia could modulate many of the protective pathways involved in the protection of the intestine and distant organs, including inflammatory cells infiltration, oxidative and nitrosative stress, and energy metabolism.

These findings support the potential application of rescue moderate hypothermia in clinical conditions associated with mesenteric reperfusion injury in adult patients, such as acute mesenteric arterial occlusion. However, some evidence exists to suggest that the response to intestinal I/R of mature animals differs to some extent from that of developing animals. For instance, sustained hypoperfusion of the intestine followed by normoperfusion induces a marked impairment of vascular response to endothelin-1 in 3-day old piglets, resulting in an increase in vascular resistance and a reduction in both oxygen delivery and consumption in the mesenteric circulation (Nankervis et al, 2000). However, 35-day old piglets are significantly more resistant to mesenteric hypoperfusion, and can maintain a normal level of oxygen consumption due to a reduced mesenteric vascular resistance and increased oxygen delivery. Similarly, there are some significant differences in the pattern and severity of evolution of MODS in adult and paediatric patients. For example, adult patients with severe sepsis initially experience severe cardiopulmonary compromise, which is then followed by pulmonary, renal and hepatic impairment (Brun-Buisson et al, 1995). In contrast, severely septic paediatric patients develop sequential respiratory, hepatic and renal failure, with cardiovascular collapse usually ensuing as a consequence of failure of the other organs (Doughty et al, 1998).

Using a model of 11-13 day old rats, I demonstrated that mesenteric reperfusion elicits both intestinal injury and impairment of distant organs (including lungs and liver), eventually resulting in death of experimental animals. The metabolic pathways involved in the development of such MODS are comparable to those observed in adult animals, including neutrophil infiltration and oxidative stress in intestine and lungs, systemic overproduction of nitric oxide, and hepatic energy failure (see Chapters 4 and 6). When applied at the onset of reperfusion, hypothermia proved to modulate these pathways in infant rats to a

similar extent as in adult animals, and results in a significant improvement in mortality that persists after rewarming (see Chapter 6).

In a separate set of experiments using this infant model of mesenteric reperfusion injury, I tested whether inhibition of the harmful free radical peroxynitrite by administration of a selective decomposition catalyst (FeTMPyP) at reperfusion could lead to improvement of intestinal injury and protection to distant organs (see chapter 5). FeTMPyP proved to be effective in preventing oxidative and nitrosative stress in the ileum and distant organs, confirming the significant role of peroxynitrite in the pathogenesis of reperfusion injury. Our results suggest that this protection is mediated at least in part by inhibition of P-Selectin upregulation and neutrophil recruitment in the intestine, although other pathways are likely to be implicated. The results of this and other studies appear encouraging in suggesting a role for selective peroxynitrite decomposition catalysts in ameliorating reperfusion injury.

After establishing the effects of hypothermia and peroxynitrite decomposition catalyst FeTMPyP in an animal model of mesenteric reperfusion injury, I investigated the pattern of the inflammatory response in the intestine of patients with NEC. Few studies have been published to date characterising the intestinal inflammatory process in human NEC, although it is generally accepted that an excessive activation of systemic inflammation plays a crucial role in the pathogenesis of MODS in this disease. As previously discussed, the beneficial effects of both whole-body hypothermia and peroxynitrite decomposition catalyst FeTMPyP appeared to be mediated, at least in part, via blunting of the inflammatory response in the intestine. Description of the inflammatory process in the gut of newborns with NEC, in particular inflammatory cell infiltrate and adhesion molecule expression, reinforces the relevance of the experimental findings in our animal model in view of a potential clinical application of these therapeutic strategies. My studies on intestinal specimens from human NEC confirmed the importance of both neutrophil and macrophage infiltration in determining tissue injury, which appears to be mediated via endothelial expression of P-Selectin and ICAM-1. Thus, P-selectin expression and neutrophil infiltration appear to play a central role in human NEC as well as in animal intestinal I/R

injury. This suggests that therapeutic strategies which I showed to be beneficial in an animal model of NEC may also warrant investigation in human disease in order to prevent P-Selectin activation and neutrophil infiltration in the intestine. Furthermore, blunting of the inflammatory response in the gut could prevent the generation of a systemic inflammatory response and the subsequent development of MODS as previously discussed.

Interestingly, upregulation of E-selectin on intestinal vascular endothelium proved to be strongly associated with the failure of multiple organs and poor outcome that characterised fulminate NEC. Such E-Selectin upregulation is known to be mediated via overproduction of ROS and RNS, as discussed in Chapter 7 (Ichikawa et al, 1997; Russell et al, 2000). Although I was unable to characterise E-Selectin expression in intestinal specimens from our experimental animals undergoing intestinal I/R because of the absence of suitable antibodies, it may be speculated that the observed protection against oxidative and nitrosative stress by both hypothermia and FeTMPyP would result in inhibition of E-Selectin upregulation. This would further support the potential clinical application of both hypothermia and FeTMPyP, regardless of whether E-Selectin overexpression represents a simple marker of rapidly evolving NEC or it plays an active role in promoting systemic inflammation.

9.2 FUTURE WORK

As discussed in the previous section, I have provided convincing evidence for a potential use of both whole-body hypothermia and FeTMPyP as a rescue therapy for human conditions associated with intestinal I/R injury, and particularly NEC.

FeTMPyP application has so far been limited to pre-clinical trials, and long-term clinical studies are therefore necessary before their safety for use in paediatric patients is established. On the other hand, therapeutic whole-body hypothermia has been used in a large number of neonatal patients for the treatment of intrapartum asphyxia (Jacobs et al, 2007) and other conditions (see Chapter 1), and its side effects (including haemodynamic instability and coagulopathy) appear

to be rare and controllable. This would suggest that hypothermia is perhaps the most promising of the therapeutical strategies we evaluated in our experimental studies.

Since one of the major issues with NEC patients is the severe comorbidity observed in long-term survivors, especially intestinal failure secondary to short bowel syndrome and impaired neurodevelopmental outcome (Casey et al, 2008; Rees et al, 2007), further studies should be aimed at investigating whether the protective effect of hypothermia during the acute phase also leads to a reduction in the long-term sequelae. It would be extremely interesting to establish a long-term survivable neonatal model of intestinal I/R injury, where rats are returned to their dams after a period of normothermic or hypothermic intestinal reperfusion. This would allow investigation of long-term survival and developmental outcome. Survivors could be investigated at various time points during their development, and intestinal absorption and length could be measured. Neurological outcome could also be assessed, with particular attention to visual and psychomotor functions.

Given the expanding indications of hypothermia in the treatment of various human conditions, and the robust benefits observed in experimental studies as explained in this thesis, researchers from my unit considered investigating hypothermia as an experimental treatment for neonates with necrotizing enterocolitis. However, the subjects of clinical trials and preliminary studies of safety and efficacy of moderate hypothermia previously published concerned full term infants, and no data were available regarding the safety of therapeutic moderate hypothermia when applied to infants born prematurely or with very low birth weight. Before the potential clinical benefits of moderate hypothermia on patient outcome could be assessed in a randomised controlled trial, it was first necessary to demonstrate that this intervention is feasible and that it does not have obvious adverse consequences in a cohort of preterm infants with NEC and MODS.

After completion of the research described in this thesis, investigators from my same group have therefore designed a pilot study to evaluate the feasibility and

safety of hypothermia in preterm neonates with advanced necrotizing enterocolitis (Hall et al, 2010).

A prospective, nonrandomized pilot study was designed. Fifteen neonates who were referred for surgical management of acute NEC at Great Ormond Street Hospital, London, between October 2004 and October 2006 were studied. A historical comparison group of 10 infants who were admitted immediately before (April 2004 to October 2004) commencement of the study served as a control group. All infants with a clinical diagnosis of NEC (pneumatosis intestinalis on abdominal radiograph and at least Bell stage II) and evidence of MODS were considered. Infants with bilateral grade 4 intraventricular hemorrhage (IVH) were excluded due to the expectedly increased risk of bleeding.

Three groups of infants were studied sequentially:

- Group 1: $35.5 \pm 0.5^{\circ}\text{C}$ core temperature for 48 hours ($n = 5$)
- Group 2: $34.5 \pm 0.5^{\circ}\text{C}$ core temperature for 48 hours ($n = 5$)
- Group 3: $33.5 \pm 0.5^{\circ}\text{C}$ core temperature for 48 hours ($n = 5$)

Whole-body cooling was achieved by ambient temperature adjustment with or without cooling mattress, and all infants were then slowly rewarmed to 37°C at the end of the cooling period.

Patients were monitored for adverse effects that have been previously associated with hypothermia (including bradycardia, hypotension, hypokalemia, thrombocytopenia, and sepsis).

Results of this study showed that whole body hypothermia can be achieved simply; lowering incubator temperature or reducing radiant warmer output was adequate to achieve target temperature in all but 2 infants, both of whom weighed >1500 g and were easily cooled with a cooling mattress. Target temperature was achieved after a median of 2.25 hours and time spent within target range was over 90%. Although this was achieved without servocontrol, this was labor-intensive and small fluctuations of temperature outside the target range were observed. The use of servocontrol in the future is recommended in order to reduce workload, improve temperature control, and limit potential adverse effects.

There were no clinically significant differences in hematologic or biochemical parameters (including haemoglobin concentration, white blood cell

and platelet count, coagulation parameters, electrolytes and renal function) throughout the study period. Sodium concentrations were statistically significantly lower in the control group compared with each of the study groups such that control infants tended to have hyponatremia.

Analysis of plasma cytokines showed that IL-1 β and IL-10 both were significantly lower during the first 24 hours of hypothermia compared with enrollment, and IL-10 but not IL-1 β was significantly lower after rewarming compared with the second 24 hours of hypothermia. IL-6 concentration did not change significantly during induction of hypothermia or during rewarming.

Although there were no statistically significant differences in blood or blood product requirements between the study and control groups, there was a trend toward higher requirements in group 3. This may reflect the temperature-dependent impairment of coagulation (including significantly longer time to initial clot formation, slower rate of clot formation, and decrease in clot strength) observed in hypothermic patients as assessed by thromboelastography.

Minor physiologic changes related to hypothermia were observed, (including a decrease in heart rate by 3.5 beats per minute per 1°C), but none had clinical consequence. No changes in extent of preexisting IVH and no new intracranial hemorrhage occurred in any study infant.

Comparison of the modified organ scores between the study and control groups suggests that a similar clinical course was followed in both groups, although this study was neither designed nor powered to determine efficacy of hypothermia. Importantly, there was neither a clinically significant increase in clinical scores indicating deterioration during hypothermia nor a rebound increase after rewarming. This is supported by the absence of a rebound increase in the levels of the inflammatory cytokine concentrations after rewarming.

Two infants in the study groups died, in both cases from underlying disease rather than hypothermia; this compares favourably with the three deaths observed in the control group as well as with the expected survival of this cohort of patients.

This pilot study demonstrated that cooling preterm infants with NEC complicated by MODS for periods of up to 48 hours is feasible and safe. A

randomized study is being designed by the same authors to investigate efficacy of hypothermia in infants with NEC, and to monitor safety.

PUBLICATIONS ARISING FROM THIS THESIS

Stefanutti G, Pierro A, Parkinson EJ, Smith VV, Eaton S.

Moderate hypothermia as a rescue therapy against intestinal ischemia and reperfusion injury in the rat.

Crit Care Med 2008; 36: 1564-72.

Stefanutti G, Pierro A, Smith VV, Klein NJ, Eaton S.

Peroxynitrite decomposition catalyst FeTMPyP provides partial protection against intestinal ischemia and reperfusion injury in infant rats.

Pediatr Res 2007; 62: 43-8.

Stefanutti G, Pierro A, Vinardi S, Spitz L, Eaton S.

Moderate hypothermia protects against systemic oxidative stress in a rat model of intestinal ischemia and reperfusion injury.

Shock 2005; 24: 159-64.

Stefanutti G, Lister P, Smith VV, Peters MJ, Klein NJ, Pierro A, Eaton S.

P-selectin expression, neutrophil infiltration, and histologic injury in neonates with necrotizing enterocolitis.

J Pediatr Surg 2005; 40: 942-8.

Stefanutti G, Vejchapipat P, Williams SR, Pierro A, Eaton S.

Heart energy metabolism after intestinal ischaemia and reperfusion.

J Pediatr Surg 2004; 39: 179-83.

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Biochem Soc Trans 2003 ; 3: 1133-6.

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